PCT WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 6 :
C12Q 1/68, C07K 16/18, G01N 33/68, C12N 15/12, C07K 14/47

A1

(11) International Publication Number: WO 99/16909

(43) International Publication Date: 8 April 1999 (08.04.99)

(21) International Application Number: PCT/US98/20777

(22) International Filing Date: 1 October 1998 (01.10.98)

(30) Priority Data:
60/060,219 1 October 1997 (01.10.97) US

(71) Applicant *(for all designated States except US)*: YALE UNIVERSITY [US/US]; 451 College Street, New Haven, CT 06510 (US).

(72) Inventors; and
(75) Inventors/Applicants *(for US only)*: LIFTON, Richard, P. [US/US]; 92 Cornwall Lane, Gailford, CT 06437 (US). SIMON, David, B. [US/US]; 66 Maple Vale Drive, Woodbridge, CT 06525 (US).

(74) Agent: ADLER, Reid, G.; Morgan Lewis & Bockius, 1800 M Street, N.W., Washington, DC 20036–5869 (US).

(81) Designated States: AU, CA, JP, US, European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE).

Published
With international search report.
Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.

(54) Title: METHODS TO MODULATE BLOOD PRESSURE AND DIAGNOSE BARTTER'S SYNDROME TYPE III

(57) Abstract

Methods of identifying agonists or antagonists of the *CLCNKB* gene and the protein it encodes are disclosed. The invention also relates to the use of such agents to treat hypertension, conditions involving abnormal fluid volume and congestive heart failure and conditions related thereto. Also provided are compositions and methods for determining whether a patient is affected by or carries a mutation in the *CLCNKB* gene or the proteins they encode. The invention also relates to specific mutated nucleic acid sequences of the *CLCNKB* gene that give rise to Bartter's Syndrome Type III.

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AL	Albania
AM	Armenia
AT	Austria
AU	Australia
AZ	Azerbaijan
BA	Bosnia and Herzegovina
BB	Barbados
BE	Belgium
BF	Burkina Faso
BG	Bulgaria
BJ	Benin
BR	Brazil
BY	Belarus
CA	Canada
CF	Central African Republic
CG	Congo
CH	Switzerland
CI	Côte d'Ivoire
CM	Cameroon
CN	China
CU	Cuba
CZ	Czech Republic
DE	Germany
DK	Denmark
EE	Estonia
ES	Spain
FI	Finland
FR	France
GA	Gabon
GB	United Kingdom
GE	Georgia
GH	Ghana
GN	Guinea
GR	Greece
HU	Hungary
IE	Ireland
IL	Israel
IS	Iceland
IT	Italy
JP	Japan
KE	Kenya
KG	Kyrgyzstan
KP	Democratic People's Republic of Korea
KR	Republic of Korea
KZ	Kazakstan
LC	Saint Lucia
LI	Liechtenstein
LK	Sri Lanka
LR	Liberia
LS	Lesotho
LT	Lithuania
LU	Luxembourg
LV	Latvia
MC	Monaco
MD	Republic of Moldova
MG	Madagascar
MK	The former Yugoslav Republic of Macedonia
ML	Mali
MN	Mongolia
MR	Mauritania
MW	Malawi
MX	Mexico
NE	Niger
NL	Netherlands
NO	Norway
NZ	New Zealand
PL	Poland
PT	Portugal
RO	Romania
RU	Russian Federation
SD	Sudan
SE	Sweden
SG	Singapore
SI	Slovenia
SK	Slovakia
SN	Senegal
SZ	Swaziland
TD	Chad
TG	Togo
TJ	Tajikistan
TM	Turkmenistan
TR	Turkey
TT	Trinidad and Tobago
UA	Ukraine
UG	Uganda
US	United States of America
UZ	Uzbekistan
VN	Viet Nam
YU	Yugoslavia
ZW	Zimbabwe

METHODS TO MODULATE BLOOD PRESSURE AND DIAGNOSE BARTTER'S SYNDROME TYPE III

Technical Field

The invention relates to the field of modulating blood pressure by affecting salt and fluid retention. The invention also relates to the field of diagnosing patients with Bartter's Syndrome Type III. More specifically, the invention provides compositions and methods for determining whether an individual is affected by or carries a mutation in the *CLCNKB* gene that encodes a defective or absent protein involved in renal chloride ion transport.

Background of the Invention

In higher eukaryotes the maintenance of the proper ionic composition and volume of the intravascular space is critical for normal neuromuscular function and delivery of oxygen and nutrients to tissues. The kidney plays a dominant role in determining the long-term set points of fluid and electrolyte balance, and maintaining homeostasis despite wide variations in environmental exposure. Dysfunction in these components of kidney function is likely the cause of a variety of clinical disorders ranging from altered blood pressure to changes in intravascular volume that result from abnormalities in electrolyte homeostasis. Examination of Mendelian disorders of fluid and electrolyte homeostasis provides the opportunity to dissect the fundamental mechanisms governing this processed. This effort provides insight into basic physiology and also identifies targets in which more subtle variation might commonly have effects in the population (Lifton, R.P., *Proc. Nat. Acad. Sci. U.S.A.* 92: 8545-8551 (1995)).

Bartter's Syndrome and Gitelman's Syndrome

Bartter's Syndrome is an autosomal recessive disorder featuring hypokalemic metabolic alkalosis with salt wasting (Bartter, F.C., *et al.*, *Am. J. Med.* 33:811-828 (1962)). Affected patients have been shown to have a diverse array of additional metabolic abnormalities, including elevated plasma renin activity (Bartter, F.C., *et al.*, *Am. J. Med.* 33:811-828 (1962); hyperaldosteronism (Goodman, A.D., *et al.*, *N. Eng. J. Med.* 281:1435-

1439 (1969)); altered prostaglandin metabolism (Dunn, M.J., *Kid. Int.* 19:86-102 (1981)); elevated levels of atrial natriuretic peptide (Imai, M., *et al.*, *J. Ped.* 74:738-749 (1969)); Graham, R. M., *et al.*, *Hypertension* 8:549-551 (1986)); abnormal platelet function (Rodrigues Pereira, R., *et al.*, *Am. J. Med. Gen.* 15:79-84 (1983)); and insensitivity to the vasoconstrictive effects of angiotensin II and norepinephrine (Bartter, F.C., *et al.*, *Am. J. Med.* 33:811-828 (1962); and Silverberg, A.B., *et al.*, *Am. J. Med.* 64:231-235 (1978)).

Symptoms and signs of disease in affected patients reflect these diverse physiologic findings, and include signs of intravascular volume depletion (Bettinelli, A., *et al.*, *J. Pediatr.* 120:38-43 (1992)), seizures (Iwata, F., *et al.*, *Acta Paed. Japonica* 35:252-257 (1993)), tetany (Bettinelli, A., *et al.*, *J. Pediatr.* 120:38-43 (1992)), muscular weakness (Marco-Franco, J. E., *et al.*, *Clin. Neph.* 42:33-37 (1994)), paresthesias (Zarraga Larrondo, S., *et al.*, *Nephron* 62:340-344 (1992)), and joint pain with chondrocalcinosis (Smilde, T. J., *et al.*, *J. of Rheum.* 21:1515-1519 (1994)). Persistent abnormalities in electrolyte composition have resulted in stunted growth and mental retardation in some affected subjects (Simopoulos, A.P., *et al.*, *Nephron* 23:130-135 (1979)). These profound derangements in electrolyte homeostasis can lead to the misdiagnosis of bulimia and/or diuretic abuse in affected individuals (Okusa, M.D. and Bia, M. J. Bartter's Syndrome: In Hormone Resistance and Other Endocrine Paradoxes, eds. Cohen, P. and Foa, P. 231-263 (Springer Verlag, New York, 1987)).

Bartter's Syndrome was proposed to be a heterogeneous entity with at least two subsets, Gitelman's Syndrome (Gitelman, H. J., Graham, J. B., and Welt, L.G. A new familial disorder characterized by hypokalemia and hypomagnesemia. *Trans. Assoc. Am. Phys.* 79:221-235 (1966)) and "true Bartter's Syndrome" (Bettinelli, A., *et al.*, *J. Pediatr.* 120:38-43 (1992)). Gitelman's Syndrome refers to the predominant subset of patients with hypokalemic alkalosis in conjunction with hypocalciuria and hypomagnesemia, while true Bartter's Syndrome refers to patients with normal or hypercalciuria and typically normal magnesium levels.

True Bartter's patients are said to present clinically at early ages (less than 5 years) with signs of vascular volume depletion, while Gitelman's Syndrome patients typically present at older ages without overt hypovolemia (Bettinelli, A., *et al.*, *J. Pediatr.* 120:38-43

(1992)). Nonetheless, the overlapping features of these disorders has resulted in considerable confusion and controversy regarding their classification, with many patients having features of Gitelman's Syndrome being labeled as having Bartter's Syndrome in the literature (Rudin, A., *et al.*, *Scand. J. Urol. Nephrol.* 22:35-39 (1988)). The pathogenesis of these disorders has remained uncertain, with wide speculation as to which observed abnormalities are primary and which are secondary consequences of underlying primary abnormalities (Clive, D.M. *Am. J. Kid. Dis.* 25:813-823 (1995)). Presently, there is not an easy method for differentiating these disorders; differentiation being based solely on evaluating the clinical symptoms that are presented.

Gitelman's Syndrome is a genetically homogeneous autosomal recessive trait caused by loss of function mutations in the thiazide-sensitive Na-Cl cotransporter protein (TSC) located in the renal distal convoluted tubule. The predominant clinical and physiologic abnormalities seen in these patients can be explained by the resultant salt wasting from this nephron segment.

Dissection of the physiology of renal electrolyte homeostasis has identified a number of potential candidate genes for Gitelman's Syndrome and Bartter's Syndrome; prior studies have investigated genes encoding atrial natriuretic peptide and the angiotensin II receptor (AT1) (Graham, R. M., *et al.*, *Hypertension* 8:549-551 (1986), Yoshida, H., *et al.*, *Kid. Int.* 46:1505-1509 (1994)). Another attractive candidate gene is the thiazide-sensitive Na-Cl cotransporter of the distal convoluted tubule (thiazide-sensitive cotransporter, TSC), which is believed to be the principle mediator of sodium and chloride reabsorption in this nephron segment, accounting for a significant fraction of net renal sodium reabsorption (Ellison, D.H., *Ann. Int. Med.* 114:886-894 (1991)). This cotransporter is the target of thiazide diuretics, one of the major classes of agents used in the treatment of high blood pressure. cDNAs encoding the TSC have recently been cloned from flounder bladder and rat kidney (Gamba, G., *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* 90:2749-2753 (1993); and Gamba, G., *et al.*, *J. Biol. Chem.* 269:17713-17722 (1994)). The encoded protein from rat comprises 1002 amino acids, and contains twelve putative transmembrane domains, with long intracellular amino and carboxy termini. Similarities in some features of patients with Gitelman's Syndrome and patients receiving thiazide diuretics raise the possibility that mutation in

TSC, causing loss of function, could result in Gitelman's Syndrome. This consideration motivates examination of *TSC* as a candidate gene for Gitelman's Syndrome.

The foregoing observations in patients with Bartter's Syndrome and Gitelman's Syndrome left open the question of whether Bartter's Syndrome is an allelic variant of Gitelman's Syndrome or is due to mutation in a different gene. The occurrence of salt wasting, impaired urinary concentration and calcium wasting in Bartter's patients suggests a primary renal tubular defect in the thick ascending limb (TAL) of the loop of Henle (Gill, J. R., *et al.*, *Am. J. Med.* 65:766-772 (1978)). The absorptive variant of the bumetanide-sensitive Na-K-2Cl cotransporter (NKCC2 , also known as SLC12A1) is the primary mediator of sodium and chloride reabsorption in this nephron segment (Greger, R., *Physiol. Rev.* 65:760-797 (1985)), and loss of function of this cotransporter could produce many of the features seen in affected patients. Indeed, loop diuretics, specific antagonists of this cotransporter, can produce electrolyte disturbances very similar to those seen in patients with Bartter's Syndrome (Greger, R., *et al.*, *Klin. Woschenschr.* 61:1019-1027 (1991)).

cDNAs encoding NKCC2 have recently been cloned from rat (Gamba, G. *et al.*, *J. Biol. Chem.* 26:17713-17722 (1994)), rabbit (Payne, J. A., *et al.*, *Proc. Natl. Acad. Sci. (U.S.A.)* 91:4544-4548 (1994)) and mouse (Igarashi, P. *et al.*, *Am. J. Physiol.* 269:F405-F418 (1995)). A secretory variant of this cotransporter, NKCC1 (also known as SLC12A2) has also been cloned from shark (Xu, J.-C. *et al.*, *Proc. Natl. Acad. Sci. (U.S.A.)* 91:2201-2205 (1994)) and human (Payne, J. A. *et al.*, *J. Biol. Chem.* 270:17977-17985 (1995)). All members of this family have 12 putative transmembrane spanning domains and also show structural and sequence similarity to TSC. By investigation of families with Bartter's Syndrome, it has been shown that this variant of inherited hypokalemic alkalosis is caused by mutations in the gene encoding NKCC2. These findings explain the molecular basis of this disease and suggest possible clinical features of the more common heterozygous carrier state.

Previously, it had been thought that the autosomal recessive hypokalemic alkalosis with salt wasting and low blood pressure was caused by mutations in either of two genes, *TSC* and *NKCC2*. Mutations in *TSC* (locus symbol SLC12A3, sometimes referred to as *NCCT*), encoding the thiazide-sensitive Na-Cl cotransporter of the renal distal convoluted

tubule, cause Gitelman's Syndrome, featuring salt wasting and hypokalemic alkalosis associated with marked hypocalciuria and hypomagnesemia (Simon, D.B. *et al.*, *Nature Genet.* 12:24-30 (1996)). Mutations in *NKCC2* (locus symbol SLC12A1), encoding the renal bumetanide-sensitive Na-K-2Cl cotransporter of the thick ascending limb of Henle's loop (TAL), cause Bartter's Syndrome, featuring salt wasting and hypokalemic alkalosis associated with marked hypercalciuria and frequently nephrocalcinosis (Simon, D.B., *et al.*, *Nature Genet.* 13:183-188 (1996)). While Bartter's patients typically are born prematurely with polyhydramnios and show marked dehydration in the neonatal period, Gitelman's patients typically present at older ages with neuromuscular signs and symptoms (Wang, W., *et al.*, *Ann. Rev. Physiol.* 54:81-96 (1992)).

Mutations in genes whose products regulate activity of either of these cotransporters could potentially lead to similar clinical phenotypes. An apical ATP-sensitive K^+ channel has been implicated as one such regulator of the Na-K-2Cl cotransporter in the TAL (Wang, W., *et al.*, *Ann. Rev. Physiol.* 54:81-96 (1992), Giebisch, G., *Kidney Int.* 48:1004-1009 (1995)). Since K^+ levels in the TAL are much lower than levels of Na^+ and Cl^-, availability of tubular K^+ is rate limiting for cotransporter activity; K^+ entering the cell from the tubule must be "recycled" to the lumen in order to permit sustained cotransport activity. This key role of K^+ channels in the regulation of cotransporter activity is demonstrated by the ability of potassium channel antagonists to virtually abolish Na-K-2Cl cotransporter activity, Giebisch, G., *Kidney Int.* 48:1004-1009 (1995)).

An inwardly rectifying K^+ channel (IRK) bearing many features of this regulatory channel (low single channel conductance, activation by low levels of ATP and protein kinase A (PKA), and insensitivity to voltage and calcium) has been cloned (Ho, K., *et al.*, *Nature* 362:31-38 (1993)). This channel, ROMK (locus symbol KCNJ1), is the prototype of the IRK family of potassium channels, comprising two transmembrane spanning domains, and a segment homologous to the characteristic H5 pore domain. The channel contains PKA phosphorylation sites that are required for normal channel activity. Multiple ROMK isoforms encoded by the same chromosome 11 locus are generated by alternative splicing (Yano, H., *et al.*, *Mol. Pharmacology* 45:854-860 (1994); Shuck, M.E. *et al.*, *J. Biol. Chem.* 269:24261-24270 (1994)); these isoforms have been shown to be expressed in

the kidney, specifically on the apical membrane of cells of the TAL as well as more distal nephron segments (Lee, W.S., *et al.*, *Am. J. Physiol.* (Renal Fluid Electrol. Physiol.) 268:F1124-31 (1995); Boim, M.A. *et al.*, *Am. J. Physiol.* (Renal Fluid Electrol. Physiol.) 268:F1132-40 (1995); Hebert, S.C., *Kidney Int.* 48, 1010-1016 (1995)). This channel has been proposed to be involved in potassium recycling in the TAL, as well as in net renal potassium secretion in the distal nephron.

Bartter's Syndrome Types I, II and III

Bartter's Syndrome types I and II can be caused by loss of function mutations in either of two genes, encoding the bumetanide-sensitive Na-K-2CL cotransporter (*NKCC2*, locus symbol *SLC12A1*) or the ATP-sensitive K channel ROMK (locus symbol *KCNJ1*). Clinical disease results from defective renal reabsorption of sodium chloride in the thick ascending limb of Henle's loop (TAL), where approximately 30% of filtered salt is normally reabsorbed.

Two closely related chloride channels specifically expressed in mammalian kidney have recently been described (Kieferle, S. et al., Proc. Natl. Acad. Sci. (U.S.A.) 91:6943-6947 (1994). In rat, recent studies suggest expression of ClC-K1 and ClC-K2 along the nephron from the thin ascending limb of Henle's loop onward, although the relative expression of each gene in different nephron segments is debated (Vandewalle, A. et al., *Am. J. Physiol. (Renal Fluid Electrol. Physiol.)* 272:F678-688 (1995); Adachi, S., et al., *J. Biol. Chem.* 269: 17677-17683 (1994)). Human homologs of these two genes (not necessarily orthologues) are CLCNKA and *CLCNKB* and both encode proteins of 687 amino acids (Kieferle, S. et al., 1994). These channels are members of the CLC family, comprising at least nine different mammalian chloride channels. Each is believed to have 12 transmembrane domains, intracellular amino and carboxy termini, and the prototype of the family in torpedo is gated by both voltage and chloride (Jentsch et al., Bioassays 19: 117-126 (1996).

The present invention relates to the discovery that a third gene is associated with yet another subtype of Bartter's Syndrome, Bartter's Syndrome type III. This phenotype is linked to a gene on a segment of chromosome 1 containing a renal chloride channel,

CLCNKB. Examination of mutated forms of this gene reveals loss of function mutations that impair renal chloride reabsorption in the thick ascending limb of Henle's loop. As discussed below, mutations in 17 kindreds have been identified, and include large deletions, nonsense and missense mutations. Patients harboring CLCNKB mutations are characterized by hypokalemic alkalosis with salt wasting, low blood pressure, normal magnesium and hyper- or normo-calciuria; they define a distinct subset of patients with Bartter's Syndrome in which nephrocalcinosis is absent.

The present invention provides compositions and methods that can be used to identify compounds that modulate the blood pressure and salt and fluid retention of patients in need of modulation of these parameters of homeostasis. For example, such drugs would be helpful in treating hypertension, edema, nephrotic syndrome, congestive heart failure and other pathologies that involve abnormal fluid volume. Also, the present invention relates to methods to differentiate and diagnose several types of ion transport deficiencies, particularly Bartter's Syndrome type III from the other two types of Bartter's Syndrome (I and II) and from Gitelman's Syndrome in addition to differential diagnosis of Bartter's Syndrome type III and the abuse of diuretics. The present invention further provides methods and compositions that can be used to identify heterozygote carriers for Bartter's Syndrome type III. Carriers, though not displaying severe clinical symptoms, nonetheless display mild to moderate pathologies.

All published articles, patents and other publications cited throughout this application are herein incorporated by reference in their entirety, as well as U.S. provisional application 60/060,219 filed October 1, 1997. This application also relates to our recent U.S. patent applications Serial Nos. 08/778,052 and 60/040,171 (which is related to PCT application US98/04681), which are hereby incorporated by reference in their entirety(a).

Summary of the Invention

The present invention is based, in part, on the identification of the role of the human renal chloride ion transporter, CLCNKB. In one aspect, it relates to the discovery that mutant forms of the *CLCNKB* gene cause pathologies associated with salt wasting and hypotension. Thus, a related aspect of the present invention is the identification of agents,

and the associated therapeutic methods, that modulate blood pressure and for fluid and/or fluid volume by agonizing or antagonizing the CLCNKB protein in patients having normal or wild type *CLCNKB* genes.

The present invention specifically provides the amino acid sequences or associated PCR primers useful in diagnosing the various subtypes of Bartter's Syndrome type III, except for conditions in which the *CLCNKB* gene is not present, and in developing methods and agents for treating these pathologies.

The present invention also is based, in part, on the discovery that various mutant forms of the *CLCNKB* gene give rise to loss-of-function mutant forms of the protein that produce pathologies involving salt wasting and hypotension. Based on these observations, the present invention provides compositions and methods for use in identifying agonists and antagonists of the CLCNKB protein. Accordingly, the wild-type form of the CLCNKB protein and its various normal allelic variants provide a useful target for the screening of compounds that modulate the function of the normal gene product. Antagonists are contemplated as being particularly useful as diuretics and antihypertensive agents for use in treating edema, congestive heart failure, hypertension, nephrotic syndrome and other pathologies involving abnormal fluid volume.

In yet another aspect of the present invention, methods are provided for the development and clinical application of compounds that modulate the activity of the CLCNKB protein. Agonists, antagonists and other modulators expressly are contemplated. It is contemplated that antagonists to the gene will produce diuretic antihypertensives; and, conversely, agonists to the gene's expression product will function as antihypotensive agents.

In a related aspect, the present invention also includes antibodies that specifically bind to the wild type or normal CLCNKB gene products. Fragments of such monoclonal antibodies or the polyclonal antisera which contain the immunologically significant portion can be used as antagonists, as well as the intact antibodies. Use of humanized antibodies and immunologically reactive fragments, such as the Fab, Fab', of $F(ab')_2$ fragments is often preferable, especially in a therapeutic context, as these fragments are generally less immunogenic than the whole immunoglobulin. Use of such antibodies or of antisense

molecules that hybridize to *CLCNKB* gene and transcripts as agonists or antagonists is also contemplated.

Brief Description of the Drawings

Figure 1. Organization of the *CLCNKA* and *CLCNKB* genes

A. Intron-exon organization of *CLCNKA* and *CLCNKB*.

Except as noted, CLCNKA and CLCNKB have an identical organization, and a single schematic diagram is shown. The gray boxes depict the coding exons, and the thin horizontal line depicts non-coding genomic DNA. The codon number and position in the codon of the first base of each coding exon is indicated; for example, the symbol 34_2 at the start of exon 2 indicates that the first base of this exon is the second base of codon 34. Above the gene, the estimated sizes of the introns are indicated; where indicated, symbols A and B denote the different sizes of the introns in CLCNKA and CLCNKB, respectively. Genomic sequence data for CLCNKA and CLCNKB has been deposited in Genbank (Accession Numbers: Z230643 and Z30664, respectively).

B. Topographic relationship of CLCNKA and CLCNKB.

Topographic relationship of CLCNKA and CLCNKB. A schematic diagram of the two genes is shown. Above the schematic, the location of cleavage sites for endonuclease BglII is indicated, with the distance in kilobase pairs indicated. The two genes are organized in the same transcriptional orientation, with CLCNKA 5' of CLCNKB; the distance between the two genes is 11 kb.

C. Localization of the *CLCNK* locus on the human genetic map.

Localization of the CLCNK locus on the human genetic map. The results of multipoint linkage comparing segregation of marker CLCKA13 to segregation of other chromosome 1 loci in CEPH kindreds is shown as the LOD score plotted against map position. The location of marker loci on the map is indicated, and the 1000:1 support interval for the location of the CLCNK locus is indicated by the horizontal bar at the top of the figure.

Figure 2. Evidence for linkage of Bartter's Syndrome to CLCNK.

Family relationships in 5 consanguineous Bartter's kindreds are shown. Affected subjects are indicated by filled symbols "#" or "!". Genotypes at marker loci tightly linked to CLCNK are shown. These five kindreds show homozygosity at all loci tightly linked to CLCNK.

Figure 3. Homozygous loss of *CLCNKB* in Bartter's Syndrome type III patients.

A-C. Primers specific for CLCNKB were used to direct PCR using DNA of normal subjects or index cases of unrelated Bartter's kindreds as template. The products of PCR were fractionated by electrophoresis on an 1% agarose gel. The identity of the template DNA in each reaction is indicated at the top of each lane ("nl" indicates genomic DNA of an unaffected normal control). As a positive control, primers specific for exon 1 of an unrelated gene, *NCCT*, was included in each reaction to ensure that absence of product was not simply due to PCR failure. **(A)** Amplification using forward and reverse primers in exons 1 and 2 (hCLCNKBex1-2 in Figure 9). (B) Amplification using forward and reverse primers in exons 6 and 9 (hCLCNKBex6-9 in Figure 9). (C) Amplification using forward and reverse primers in exons 17 and 19 (hCLCNKBex17-19 in Figure 9).

D-E. Primers that amplify exons 8 (panel D) and 7 (panel E) of both CLCNKA and CLCNKB were used to direct PCR, and the radioactive products were analyzed by SSCP. The respective products arising from CLCNKA and CLCNKB are indicated. In **panel (E)**, the index case of two kindreds (BAR238-1 and BAR251-1) is shown along with their parents (238-2 and 3, 251-2 and 3) and one unaffected sibling (238-4).

Figure 4. Loss of *CLCNK* sequences by Southern blotting.

Genomic DNA of index cases of indicated kindreds was digested with BglII, fractionated by electrophoresis on 0.7% agarose gel, denatured, and transferred to nylon membrane. ^{32}P-labeled CLCNKB probes corresponding to either exons 10-17 (panel A) or exons 3-9 (**panel B**) of the CLCNKA cDNA were hybridized to the filter. In **panel A**, normal subjects are seen to yield a single fragment of 9.5 kb in length which corresponds to the fragment bearing exons 10-17 of CLCNKB; CLCNKA shows an RFLP, with two

allelic fragments of 8.5 and 6.5 kb. In kindred BAR276, which by PCR has a partial deletion, the open arrow indicates the position of an aberrant fragment that is consistent with the product of partial deletion. In panel B, normal subjects yield a 2.0 kb fragment from CLCNKB and a 2.8 kb fragment from CLCNKA.

Figure 5. CLCNKA and CLCNKB exons deleted in Bartter's syndrome type III patients.

This data shows the loss of exons in the members of kindreds that express Bartter's syndrome type III. The majority of these members show a complete deletion of the *CLCNKB* gene. Three individuals have selected deletion of several exons which has led to loss of function of CLCNKB (BAR242, BAR276 and BAR226).

Figure 6. Unequal crossover and point mutations in Bartter's Syndrome type III patients.

A. Unequal crossover resulting in deletion in kindred BAR226.

(i) PCR was performed using a forward primer specific for CLCNKA and a reverse primer specific for CLCNKB; the products were fractionated on agarose gel. The two affected cases and their parents yield the predicted 2.0 kb product from unequal crossing over, while none of 80 unrelated normal subjects yield a product using these primers. The sequence of this product confirms its chimeric nature.

(ii) Schematic diagram of unequal crossing over as the origin of the chimeric gene in BAR226; one of the progeny chromosomes of unequal crossing over has a single chimeric gene fusing 5' CLCNKA sequences to the more distal CLCNKB sequences.

B. Missense and nonsense mutations in CLCNKB in Bartter's syndrome type III patients.

Variants were identified by SSCP and subjected to DNA sequence analysis. Representative examples of autoradiographs are shown at the top of each panel, and the corresponding DNA sequence of the sense strand of mutant (**left**) and wild-type alleles (**right**) are shown at the bottom of each panel. Kindred numbers are indicated, and subjects with Bartter's syndrome are indicated by asterisks, "*". The symbol "nl" represents unrelated normal subjects. Arrows indicate variants specific for Bartter's

syndrome kindreds. Variant bases are indicated by an asterisk, "*", above the sequence figures.

(i) A heterozygous single base substitution changes codon GCT at A349 to GAT at D349 in BAR282. Analysis of the parents (subjects 2 and 3) indicates transmission from subject 2, the mother.

(ii) A heterozygous substitution changes codon CAG at Q513 to UAG Stop513 in BAR283.

(iii) A homozygous missense mutation changes GCG at A204 to ACG at T204 in kindreds BAR184 and BAR205.

(iv) A homozygous missense mutation changes codon CCG P124 to CTG L124 in BAR274 and BAR294.

Figure 7. Mutations and Functional Schematic

A. Location of point mutations in *CLCNKB* in patients with Bartter's Syndrome type III.

A schematic diagram of *CLCNKB* is shown and depicted as spanning the plasma membrane twelve times (Jentsch, T. J. *et al.*, (1996) *Bioessays* 19:117-126). The locations and consequences of mutations identified in Bartter's patients are identified.

B. Physiology of NaCl reabsorption in the renal thick ascending limb (TAL).

A schematic diagram of a TAL cell is shown. NaCl enters the cell from the lumen of the renal tubule across the apical membrane via the Na-K-2Cl cotransporter (the gene product of *NKCC2*). K^+ is recycled into the lumen via the K^+ channel ROMK to permit reabsorption of a large fraction of NaCl entering the TAL. Exit of Cl^- across the basolateral membrane is believed to be via a Cl^- channel, while Na^+ exits via the Na/K-ATPase. Evidence herein demonstrates that CLCNKB is a principal mediator of chloride reabsorption in the TAL.

Figure 8. Clinical characteristics of index cases with Bartter's syndrome.

Details the medical history of the subjects suffering from Bartter's Syndrome including: the age of diagnosis (Dx) in years; serum potassium levels (mM, nl 3.5-5.0); "HCO_3" is serum bicarbonate (mM, nl 22-28), "U_{Ca}/U_{Cr}", urinary calcium:creatine ratio (mM:mM, nl 0.2-0.4). "Nephrocal." is nephrocalcinosis determined by ultrasonography. "*" indicates the presence of a homozygous mutation. "nl" refers to normal range.

Figure 9. PCR Primers for Analysis of the Human *CLCNKB* Gene.

SSCP primers are all within introns with the exception of: hCLCNKBex1-forward which lies proximal to the initiation codon and hCLCNKBex19-reverse which lies distal to the normal termination codon. "*" indicates *CLCNKB* specific primers; other primer pairs amplify both *CLCNKA* and *CLCNKB*. Long-range PCR primers all lie within CLCNKB exons.

Figure 10. Nucleotide and Amino Acid Sequences of CLCNKB.

A. Nucleotide sequence of *CLCNKB*

The *CLCNKB* nucleic acid sequence was published in Kieferle, S. *et al.*, (1994), (*Proc. Natl Acad. Sci. U.S.A.*), 91:6943-6947; the Genbank accession number is Z30644.

B. Amino Acid sequence of CLCNKB

The CLCNKB protein is 687 amino acids long. The Genbank accession number is 521074.

C. Mutations in CLCNKB in patients afflicted with Bartter's Syndrome type III

This table lists the single nucleotide mutations found in 5 patients that change the CLCNKB nucleic acid and protein sequences from the wild-type sequences shown in **Figure 10A** and **Figure 10B** respectively. The nucleic acid residue is denoted by the number adjacent to the residue. The amino acid residue number that is changed is denoted by the number adjacent to the residue.

Modes of Carrying Out the Invention

I. General Description

The present invention is based, in part, on the identification of the effects of mutant forms and deletion mutants of the human renal chloride ion transport protein, CLCNKB, that result in pathological conditions associated with abnormal ion transport, particularly Bartter's Syndrome type III. Some aspects of the present invention are disclosed in a publication by the authors, which is herein incorporated by reference: Simon, D.B., *et al., Nature Genet.* 17: 171-178 (1997). The present invention specifically provides the full or partial amino acid sequence of mutants or variants of the CLCNKB protein, as well as the corresponding nucleotide sequences. The proteins and nucleic acid molecules can be used to identify agents that modulate the activity of the normal CLCNKB protein, and in diagnosing ion transport disorders, distinguishing between the different ion transport disorders, and in developing methods and agents for treating these pathologies. In particular, PCR primers are provided that can be used to identify patients who have a deletion mutant in which the *CLCNKB* gene is not present. The invention also provides for a method of identifying compounds that modulate hypertension (*e.g.*, essential hypertension) and conditions with congestive heart failure by isolating agents which modulate the *CLCNKB* gene or the protein it encodes.

II. Specific Embodiments

A. CLCNKB Protein

Prior to the present invention, two related chloride channels had been identified that are specifically expressed in mammalian kidney (Kieferle, S., *et al.*, (1994) *Proc. Natl Acad. Sci. (U.S.A.)* 91:6943-6947). Thus, in rat, recent studies suggest expression of ClC-K1 and ClC-K2 along the nephron from the thin ascending limb of Henle's loop onwards, although the relative expression of each gene in different nephrons is debated (Vadewalle, A., *et al.*, (1995) *Am. J. Physiol. (Renal Fluid Electrol. Physiol.)* 272:F678-688); Adachi, S., *et al.*, (1994) *J. Biol. Chem.* 269: 17677-17683). Human genes that are homologous to these two genes are called *CLCNKA* and *CLCNKB*, and both encode proteins of 687 amino acids (Genbank Accession Nos. 521072 and 521074, respectively). These specific chloride channels are members of the ClC family, comprising at least nine mammalian chloride channels. Each is believed to have twelve transmembrane domains and

intracellular amino and carboy termini, and the prototype of the family in torpedo is gated by both voltage and chloride (Jentsch, T. J. *et al.*, *Bioessays* 19:117-126 (1996)).

Prior to this invention, no one had characterized naturally occurring human wild-type variants of the CLCNKB proteins; and no one had characterized human mutants and altered variants of CLCNKB proteins. The present invention provides the full or partial amino acid sequences of allelic variants of wild-type CLCNKB protein and altered variants (**Figure 10C**) of the human CLCNKB protein that give rise to ion transport deficiencies, or describes the particular mutation or mutations involved, as well as providing related nucleotide sequences, particularly primers useful in PCR-related diagnostic techniques.

Thus, in one embodiment, the present invention provides the ability, using PCR techniques and primers disclosed in **Figure 9** to identify particular mutants (**Figure 10C**) and altered variants of the human CLCNKB gene and their associated protein products (**Figure 6B**) proteins using cloned or synthesized nucleic acid molecules described herein.

As used in this specification, the following terms has the ascribed meanings:

The term "wild-type human CLCNKB proteins" includes all naturally occurring allelic variants of the human CLCNKB protein that possess normal CLCNKB activity. In general, wild-type allelic variants of the CLCNKB protein generally will have a slightly different amino acid sequence than that specifically provided in Genbank Seq. ID No. 521074. Allelic variants, though possessing a slightly different amino acid sequence than those recited above, nevertheless will posses the ability to be an ATP sensitive K^+ transporter. Typically, allelic variants of the wild-type CLCNKB protein will contain conservative amino acid substitutions from the wild-type sequences or will contain a substitution of an amino acid from a corresponding position in a CLCNKB homologue (a CLCNKB protein isolated from an organism other than human).

The term "mutated or altered human CLCNKB protein" refers to a protein that has the amino acid sequence of a mutated or altered allelic variant of human CLCNKB. The mutated or altered CLCNKB proteins of the present invention include those specifically identified and characterized herein (**Figure 6B**), as well as other mutants and allelic variants that can be isolated and characterized without undue experimentation following

the methods outlined below. For the sake of convenience, all of the mutated or altered human CLCNKB proteins of the present invention will be collectively referred to as mutated or altered CLCNKB proteins.

Additionally, the term "mutated or altered human CLCNKB protein" includes all naturally occurring allelic variants of the human CLCNKB protein that do not posses normal CLCNKB activity. In general, mutated or altered allelic variants of the CLCNKB protein may/will have a slightly to a radically different amino acid sequence than that specifically provided in Genbank Seq. ID No. 521074 for the herein-described wild-type CLCNKB protein. Mutated or altered allelic variants will be not be able to transport one or more of the ions that are transported by wild-type CLCNKB, or will be unable to transport such ions at normal or wild-type levels. Typically, allelic variants of the mutated or altered CLCNKB protein will contain: non-conservative amino acid substitutions from the wild-type sequences herein described, a substitution of an amino acid other than the amino acid found in a corresponding position in a CLCNKB homologue (a CLCNKB protein isolated from an organism other than human), a frame shift mutation, an insertion of a stop codon, or a deletion or insertion of one or more amino acids into the CLCNKB sequence. In some kindreds, the relevant mutation is the deletion of the *CLCNKB* gene.

The CLCNKB proteins of the present invention (wild-type and mutated variants) are preferably utilized in isolated form. As used herein, a protein is said to be isolated when physical, mechanical or chemical methods are employed to remove the CLCNKB protein from cellular constituents that are normally associated with the protein. A skilled artisan can readily employ standard purification methods to obtain an isolated CLCNKB protein. The nature and degree of isolation will depend on the intended use.

The cloning of CLCNKB encoding nucleic acid molecules makes it possible to generate defined fragments of the CLCNKB proteins of the present invention. As discussed below, fragments of the CLCNKB proteins of the present invention are particularly useful in generating domain specific antibodies, in identifying agents that bind to a CLCNKB protein and in identifying CLCNKB intra- or extracellular binding partners. In particular, use of the CLCNKB protein permits the identification of compounds that agonize or antagonize the CLCNKB protein, in its normal and mutant forms.

Fragments of the CLCNKB proteins can be generated using standard peptide synthesis technology and the amino acid sequences disclosed herein. Alternatively, recombinant methods can be used to generate nucleic acid molecules that encode fragments of the CLCNKB proteins. **Figures 6B** (in the figure discussion) and **8** identify amino acid residues that are altered from wild-type residues in the altered variants of the CLCNKB proteins herein described. Fragments containing these residues/alterations are particularly useful in generating altered variant specific anti-CLCNKB antibodies.

As described below, uses of the CLCNKB proteins (*i.e.*, either wild-type or mutant) include, but are not limited to: (1) a target to identify agents that block or stimulate CLCNKB activity; (2) a target or bait to identify and isolate binding partners that bind a CLCNKB protein; (3) a method of identifying agents that block or stimulate the activity of a CLCNKB protein; and (4) an assay target to identify CLCNKB mediated activity or disease.

B. Anti-CLCNKB Antibodies

The present invention further provides antibodies that selectively bind a CLCNKB protein of the present invention. The most preferred antibodies will bind to a particular altered variant of a CLCNKB protein, but not to a wild-type variant or will bind to a wild-type variant of a CLCNKB protein, but not to an altered variant. Anti-CLCNKB antibodies that are particularly contemplated include monoclonal and polyclonal antibodies, as well as fragments containing the antigen-binding domain and/or one or more complement determining regions (*e.g.*, Fv, Fab, Fab', $F(ab')_2$ and svFv).

Antibodies are generally prepared by immunizing a suitable mammalian host (*e.g.*, mice, chickens, rabbits, goats, or horses) using a CLCNKB protein, or polypeptide fragment, thereof or an isolated or immunoconjugated variant (Harlow, E., *et al.*, Antibodies: A Laboratory Manual, Cold Spring Harbor Press, NY (1988)). Variants from patients BAR242, BAR276 and BAR226 are illustrative examples of the protein sequences that could be utilized to distinguish between the wild-type CLCNKB from one of its altered forms. Fragments containing these residues are particularly suited in generating wild-type or mutated-variant specific anti-CLCNKB antibodies. By an

alteration specific antibody, we mean an antibody that recognizes a mutant (or non-wild type) form of the CLCNKB gene.

Methods for preparing a protein for use as an immunogen and for preparing immunogenic conjugates of a protein with a carrier such as BSA, KLH, or other carrier proteins are well known in the art. In some circumstances, direct conjugation using, for example, carbodiimide reagents may be used; in other instances, linking reagents such as those supplied by Pierce Chemical Co., Rockford, IL, may be effective. For more detailed description of the carrier conjugation, refer to Harlow, E., *et al.*, Antibodies: A Laboratory Manual, Cold Spring Harbor Press, NY (1988).

Administration of the CLCNKB immunogen is conducted generally by injection over a suitable time period and with the use of a suitable adjuvant (*e.g.*, Freund's Complete Adjuvant or Freund's Incomplete Adjuvant), as is generally understood in the art. During the immunization schedule, titers of antibodies can be taken to determine adequacy of antibody formation.

It is contemplated that antibodies can be used as CLCNKB antagonists. While the polyclonal antisera produced as described above may be satisfactory for some applications, for pharmaceutical compositions, monoclonal antibody preparations are preferred. Immortalized cell lines which secrete a desired monoclonal antibody may be prepared using the standard method of Köhler and Milstein or modifications which effect immortalization of lymphocytes or spleen cells, as is generally known (Köhler, G. And C. Milstein, (1975) *Nature* 256: 495-497; Köhler, G. And C. Milstein, (1976) *Eur. J. Immunol.* 6:511-519; Köhler, G., *et al.*, (1976) *Eur. J. Immunol.* 6:292-295). The immortalized cell lines secreting the desired antibodies are screened by immunoassay (*e.g.*, ELISA) in which the antigen is the CLCNKB protein or polypeptide peptide fragment thereof. When the appropriate immortalized cell culture secreting the desired antibody is identified, the cells can be cultured either *in vitro* or by production in ascites fluid.

The desired monoclonal antibodies are then recovered from the culture supernatant or from the ascites supernatant. Fragments of the monoclonals or the polyclonal antisera which contain the immunologically significant portion can be used as antagonists, as well

as the intact antibodies. Use of immunologically reactive fragments, such as the Fab, Fab', of $F(ab')_2$, or svFv fragments is often preferable, especially in a therapeutic context, as these fragments are generally less immunogenic than the whole immunoglobulin.

The antibodies or fragments may also be produced, using current technology, by recombinant means. Regions that bind specifically to the desired regions of the transporter can also be produced in the context of chimeric or CDR grafted antibodies of multiple species origin (*e.g.*, chimeric antibodies).

As described below, anti-CLCNKB antibodies are useful as modulators of CLCNKB activity, are useful in immunoassays for detecting CLCNKB expression/activity, and for purifying wild-type and altered variants of the CLCNKB proteins. They may also be useful as antagonists for CLCNKB.

C. CLCNKB Encoding Nucleic Acid Molecules

As described above, the present invention is based, in part, on the use of nucleic acid molecules isolated from humans that encode altered mutants of wild type CLCNKB proteins. Accordingly, the present invention further provides nucleic acid molecules that encode the disclosed altered or mutant variants of the CLCNKB proteins (refer to **Figure 6B**), preferably in isolated form. For convenience, unless greater specificity is intended by the relevant text, all CLCNKB encoding nucleic acid molecules will be referred to as CLCNKB encoding nucleic acid molecules, the *CLCNKB* genes, or *CLCNKB*.

As used herein, a "nucleic acid molecule" or "nucleotide" is defined as an RNA or DNA molecule that encodes a peptide as defined above, or is complementary to a nucleic acid sequence encoding such peptides. Particularly preferred nucleic acid molecules will have a nucleotide sequence identical to or complementary to the human cDNA sequences herein disclosed. Specifically contemplated are genomic DNA, cDNA, recombinant RNA (rRNA), mRNA and antisense molecules, as well as nucleic acids based on an alternative backbone or including alternative bases whether derived from natural sources or synthesized. Such nucleic acid molecules, however, are defined further as being novel and not obvious over any prior art nucleic acid molecules encoding human or non-human homologues of CLCNKB.

As used herein, a nucleic acid molecule is said to be "isolated" when the nucleic acid molecule is substantially separated from contaminant nucleic acid encoding other polypeptides. A skilled artisan can readily employ nucleic acid isolation procedures to obtain a CLCNKB encoding nucleic acid molecule. See Sambrook *et al.*, Molecular Cloning, A Laboratory Manual, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY (1989).

The present invention further provides fragments of the CLCNKB encoding nucleic acid molecules of the present invention. As used herein, a "fragment" refers to a small portion of the entire protein encoding sequence. The size of the fragment will be determined by the intended use. For example, if the fragment is chosen so as to encode an active portion of the CLCNKB protein, such an intracellular or extracellular domain, then the fragment will need to be large enough to encode the functional region(s) of the CLCNKB protein. If the fragment is to be used as a nucleic acid probe or PCR primer, then the fragment length is chosen so as to obtain a relatively small number of false positives during probing/priming.

Fragments of the CLCNKB encoding nucleic acid molecules of the present invention (*i.e.*, synthetic oligonucleotides) that are used as probes or specific primers for the polymerase chain reaction (PCR), or to synthesize gene sequences encoding CLCNKB proteins, can easily be synthesized by chemical techniques, for example, the phosphotriester method of Matteucci, *et al.*, *J Am Chem Soc* (1981) 103:3185-3191 or using automated synthesis methods. Particularly contemplated are the PCR primer sequences of **Figure 9**. In addition, larger DNA segments can readily be prepared by well known methods, such as synthesis of a group of oligonucleotides that define various modular segments of the *CLCNKB* gene, followed by ligation of oligonucleotides to build the complete modified *CLCNKB* gene.

The CLCNKB encoding nucleic acid molecules of the present invention may further be modified so as to contain a detectable label for diagnostic and probe purposes. As described above, such probes can be used to identify nucleic acid molecules encoding other allelic variants of wild-type or altered CLCNKB proteins and as described below, such probes can be used to diagnosis the presence of mutant genes or the absence of the

CLCNKB gene as means for diagnosing a pathological condition caused by abnormal ion transport associated with mutations or deletions in the *CLCNKB* gene. A variety of such labels are known in the art and can readily be employed with the nucleotide molecules herein described. Suitable labels include, but are not limited to, biotin, radiolabeled nucleotides, and the like. A skilled artisan can employ any of the art known labels to obtain a labeled CLCNKB encoding nucleic acid molecule.

D. Isolation of Other Wild-Type and Mutant or Altered Forms of CLCNKB Encoding Nucleic Acid Molecules

As described above, the identification of the role played by mutant forms of the CLCNKB protein in the pathology/severity of ion transport-mediated deficiencies has made possible the identification of other mutants and variants of the wild-type CLCNKB, that confer a pathology associated with abnormal ion transport. These observations allows a skilled artisan to isolate nucleic acid molecules that encode other mutants of the CLCNKB proteins, in addition to the particular mutations and corresponding DNA sequences herein described.

Essentially, a skilled artisan can readily use the amino acid sequence of the human CLCNKB protein and its variants to generate antibody probes to screen expression libraries prepared from cells. Typically, polyclonal antiserum from mammals such as rabbits immunized with the purified protein (as described below) or monoclonal antibodies can be used to probe a human cDNA or genomic expression library, such as 8gtll library, prepared from a normal or effected individual, to obtain the appropriate coding sequence for wild-type or altered variants of the CLCNKB protein. The cloned cDNA sequence can be expressed as a fusion protein, expressed directly using its own control sequences, or expressed by construction using control sequences appropriate to the particular host used for expression of the enzyme. Regions implicated in the activity of CLCNKB are the preferred targets for the production of probe, diagnostic, and therapeutic antibodies. For detailed discussion of these methods, refer to E. Harlow and D. Lane (1988).

Alternatively, a portion of the CLCNKB encoding sequence herein described can be synthesized and used as a probe to retrieve DNA encoding a member of the CLCNKB family of proteins from individuals that have normal ion transport or from individuals suffering from a pathological condition that is a result of abnormal ion transport. Oligomers of approximately 18-20 nucleotides (encoding about a 6-7 amino acid stretch) are prepared and used to screen genomic DNA or cDNA libraries to obtain hybridization under stringent conditions or conditions of sufficient stringency to eliminate an undue level of false positives. This method can be used to identify and isolate altered and wild-type variants of the *CLCNKB* encoding sequences. By comparing the *CLCNKB* sequence of a patient having abnormal (or suspected abnormal renal function with the normal *CLCNKB* sequence, other mutant forms of this gene can be identified.

Additionally, pairs of oligonucleotide primers can be prepared for use in a polymerase chain reaction (PCR) to selectively amplify/clone a *CLCNKB*-encoding nucleic acid molecule, or fragment thereof. A PCR denature/anneal/extend cycle for using such PCR primers is well known in the art and can readily be adapted for use in isolating other *CLCNKB* encoding nucleic acid molecules. **Figure 9** identifies oligonucleotides of *CLCNKB* that are particularly well suited for use as a probe or as primers to diagnose Bartter's Syndrome Type III. **Figure 10C** shows the 6 nucleic acid mutations found in 5 patients (Patient medical backgrounds are summarized in **Figure 8**) and their corresponding effect in the CLCNKB protein sequence. In general, the preferred primers will flank one or more exons of the *CLCNKB* encoding nucleic acid molecule.

E. Methods for Identifying Pathological Conditions Involving Abnormal Ion Transport

The present invention further provides methods for identifying cells and individuals expressing active and altered mutants and variants of the renal chloride ion transport protein, CLCNKB, and differentiating them from patients with mutations in the Na-K-2Cl cotransporter NKCC2, the renal thiazide-sensitive Na-Cl cotransporter, TSC, and the ATP-sensitive potassium channel, ROMK. Such methods would also be helpful in the differential diagnosis of diuretic abuser as opposed to an individual suffering from

Bartter's Syndrome type III. Patients suffering from Bartter's Syndrome (types I-III) have frequently been misdiagnosed as diuretic abusers. Such methods can be used to diagnose biological and pathological processes associated with altered ion transport, particularly various variants of Bartter's Syndrome (*e.g.*, types I, II and III) and Gitelman's Syndrome, the progression of Bartter's Syndrome type III, the susceptibility of Bartter's Syndrome type III to treatment and the effectiveness of treatment for Bartter's Syndrome type III. The methods of the present invention are particularly useful in identifying carriers of the renal chloride ion transport protein CLCNKB mutations. Specifically, the presence of altered variants of the CLCNKB proteins can be identified by determining whether a wild-type or altered variant of the CLCNKB protein, or nucleic acid encoding one or more of these proteins, is expressed in a cell. The expression of an altered variant, or departure from the normal level of CLCNKB expression, can be used as a means for diagnosing pathological conditions mediated by abnormal CLCNKB activity/expression, differentiating between various ion transport deficiencies, and to identify carriers of ion transport deficiencies.

A variety of immunological and molecular genetic techniques can be used to determine if a wild-type or an altered variant of a CLCNKB protein is expressed/produced in a particular cell and/or the level at which the protein is expressed. The preferred methods will distinguish whether a wild-type or mutated from of the CLCNKB protein is expressed.

In general, an extract containing nucleic acid molecules or an extract containing proteins is prepared from cells of an individual. The extract is then assayed to determine whether a CLCNKB protein, or a *CLCNKB* encoding nucleic acid molecule, is produced in the cell. The type of protein/nucleic acid molecule expressed or the degree/level of expression provides a measurement of the nature and degree of CLCNKB activity.

For example, to perform a diagnostic test based on nucleic acid molecules, a suitable nucleic acid sample is obtained and prepared from a subject using conventional techniques. DNA can be prepared, for example, simply by boiling the sample in sodium dodecyl sulfate (SDS). Most typically for nucleic acid samples, a blood sample, a buccal swab, a hair follicle preparation or a nasal aspirate is used as a source of cells to provide

the nucleic acid molecules. The extracted nucleic acid can then be subjected to amplification, for example by using polymerase chain reaction (PCR) according to standard procedures, to selectively amplify a *CLCNKB* encoding nucleic acid molecule or fragment thereof. The size of the amplified fragment (typically following restriction endonuclease digestion) is then determined using gel electrophoresis or the fragment is sequenced (for example, see Weber and May *Am. J. Hum. Genet.* (1989) 44:388-339; Davies, J. *et al., Nature* (1994) 371:130-136). The resulting size of the fragment or sequence is then compared to the known wild-type, predicted wild-type, known altered variants and predicted altered variants of the protein in question. It must be noted, however, that in some instances, the *CLCNKB* gene is absent, and the relevant diagnostic assay must confirm its absence rather than its presence. Using these methods, the presence of wild-type or altered variants of the *CLCNKB* nucleic acid molecules and proteins can be differentiated and identified.

Alternatively, the presence or absence of one or more single base-pair polymorphism(s) (also known as single nucleotide polymorphisms, SNPs) within the CLCNKB encoding nucleic acid molecules can be determined by conventional methods which include, but are not limited to, manual and automated fluorescent DNA sequencing, selective hybridization probes, primer extension methods (Nikiforov, T. T. *et al., Nucl. Acids Res.* (1994) 22:4167-4175); oligonucleotide ligation assay (OLA) (Nickerson, D.A. *et al., Proc. Natl Acad. Sci. USA* (1990) 87:8923-8927); allele-specific PCR methods (Rust, S. *et al., Nucl. Acids Res.* (1993) 6:3623-3629); RNase mismatch cleavage, single strand conformation polymorphism (SSCP) (Orita, M. *et al., Proc. Natl Acad. Sci., USA* 86:2766-2770 (1989)), denaturing gradient gel electrophoresis (DGGE) and the like. The present diagnosis method is particularly well suited for use in biochip technologies that are being developed to be used to identify whether one of many sequence variations or SNPs is present in a sample. A skilled artisan can readily adapt any nucleic acid analytical method for use in determining whether a sample contains nucleic acid molecules that encode a wild-type or altered variant of a CLCNKB protein.

To perform a diagnostic protein-based test, a suitable protein sample is obtained and prepared from a subject using conventional techniques. Protein samples can be

prepared, for example, simply by mixing the sample with SDS, followed by salt precipitation of a protein fraction. Typically, for protein samples, a blood sample, a buccal swab, a nasal aspirate, or a biopsy of cells from tissues expressing a CLCNKB protein is used as a source of cells to provide the protein molecules. The extracted protein can then be analyzed to determine the presence of a wild-type or altered variant of a CLCNKB protein using known methods. For example, the presence of specific sized or charged variants of a protein can be identified using mobility in an electric field. Alternatively, wild-type or altered variant specific antibodies can be used. A skilled artisan can readily adapt known protein analytical methods to determine if a sample contains a wild-type or altered variant of a CLCNKB protein.

CLCNKB expression can also be used in methods to identify disorders that occur as a result of an increase or decrease in the expression of a naturally occurring *CLCNKB* gene. Specifically, nucleic acid probes that detect mRNA can be used to detect cells or tissues that express a CLCNKB protein and determine the level of such mRNA expression.

The classification of patients with recessive hypokalemic alkalosis has been problematic, however identification of mutations underlying this trait is making this classification simpler. To date, all patients with mutations in the thiazide-sensitive Na-Cl cotransporter NCCT (locus *SLC12A3*) have the phenotype of hypokalemic alkalosis, salt wasting, hypocalciuria and hypomagnesemia (Matsumoto, J., *et al.*, (1989) *Am. J. Roentgen.* 152:1251-1253; Simon, D.B., *et al.*, (1996) *Nature Genet.* 12: 4-30). We refer to these patients as having Gitelman's Syndrome. Patients with mutations in either *NKCC2*, *ROMK*, or *CLCNKB* all share the phenotype of hypokalemic alkalosis, salt wasting, and normal magnesium levels (Simon, D.B., *et al.*, (1996) *Nature Genet.* 14:152-156; Simon, D.B., *et al.*, (1996) *Nature Genet.* 13:183-188). We refer to these latter patients as Bartter's syndrome types I, II and III, respectively. While most of these patients have elevated urinary calcium:creatinine ratios (U_{Ca}:U_{Cr}), this finding appears to be variable among patients with *CLCNKB* mutations. While nephrocalcinosis is almost invariant in untreated patients with mutations in *NKCC2* and *ROMK*, this feature is absent amount patients with mutations in *CLCNKB*. It is noted that of the six consanguineous

Bartter's kindreds in which no mutations have been identified, only one of these is homozygous at loci spanning *NKCC2*, *ROMK* or *CLCNKB*. This finding indicates the presence of a mutation in at least one as yet unidentified locus causing this phenotype.

The observation that many patients have homozygous *CLCNKB* deletions indicates that these mutations result in loss of normal gene function. Physiologic and biochemical studies of these patients demonstrate a defect in chloride reabsorption in the TAL. The mechanism of the defect is presumed to be failure of normal transit of Cl^- across the basolateral membrane into the bloodstream (**Figure 7B**). The fact that mutation in this single gene can produce this phenotype indicates that there must not be other redundant pathways of chloride ion exit that can completely compensate for deficiency in this channel, and indicates therefore that despite their recent divergence, the products of the highly related genes CLCNKA and CLCNKA are not simply redundant in function in humans. This observation is further supported by the apparent absence of functional mutations altering CLCNKA in these kindreds. Similarly, while other mechanisms of chloride transit across the basolateral membrane of renal epithelia have been proposed, such as K^+-Cl^- cotransport (Greger, R., et al., (1983) Pflügers Arch. 396: 325-334), it is clear from the phenotypic effects of CLCNKB mutations that no other transporters or channels can compensate completely for loss of function of this channel.

The observation that 10 of the 17 apparently independent mutant CLCNKB alleles are deletions is of some surprise. The close linkage of the highly similar CLCNKA and CLCNKB genes suggests potential mechanisms for such loss, including gene deletion by unequal crossing over or gene conversion. Such mechanisms have been identified as the cause of mutation in several human diseases, including thalassemia (Lauer, J., *et al., Cell* (1980) 20:119-130), red-green color blindness (Nathans, J., et al., Science (1986) 232: 203:210), steroid 21-hydroxylase deficiency (White, P.C., et al., Proc. Natl Acad. Sci. (1988) 85:4436-4440) and glucocorticoid-remediable aldosteronism (Lifton, R.P., et al., Nature (1992) 355: 262-265).

We have demonstrated that the deletion seen in one kindred, BAR226, arose by unequal crossing over between CLCNKA and CLCNKB in intron 2 (refer to Figures 6A and 8). It is

possible that some of the other large deletions could also result from unequal crossing over in segments of homology beyond the 3' end of the coding region of each gene. It is noteworthy that of the 10 deletion patients, 3 of these are of Portuguese ancestry (two ascertained in Portugal, one in the USA) and 5 are African American (Figure 8).

Alternatively, as discussed in greater detail below, CLCNKB expression can also be used in methods to identify agents that increase or decrease the level of expression of a naturally occurring CLCNKB gene. For example, cells or tissues expressing a CLCNKB protein can be contacted with a test agent to determine the effects of the agent on CLCNKB expression. Agents that activate *CLCNKB* expression can be used as an agonist of CLCNKB activity, whereas agents that decrease *CLCNKB* expression can be used as an antagonist of CLCNKB activity. Antagonists of CLCNKB activity are contemplated for the treatment of conditions mediated by abnormal fluid volume. Preferred *CLCNKB* antagonists would include those that treat or modulate hypertension, congestive heart failure and conditions associated with congestive heart failure.

F. rDNA Molecules Containing an CLCNKB Encoding Nucleic Acid Molecule

The present invention further provides recombinant DNA molecules (rDNAs) that contain one or more of the wild-type or altered CLCNKB encoding sequences herein described, or a fragment of the herein-described nucleic acid molecules. As used herein, an rDNA molecule is a DNA molecule that has been subjected to molecular manipulation *in vitro*. Methods for generating rDNA molecules are well known in the art, for example, see Sambrook *et al.*, (1989). In the preferred rDNA molecules, a CLCNKB encoding DNA sequence that encodes a wild-type or altered variant of the CLCNKB protein is operably linked to one or more expression control sequences and/or vector sequences. Most preferably, the CLCNKB encoding nucleic acid molecules will encode one of the novel altered or wild-type variants herein described, for use in systems to identify agents that modulate, and more particularly that antagonize, the normal CLCNKB protein.

The choice of vector and/or expression control sequences to which one of the CLCNKB encoding sequences of the present invention is operably linked depends directly, as is well known in the art, on the functional properties desired, *e.g.*, protein expression, and the host cell

to be transformed. A vector contemplated by the present invention is at least capable of directing the replication or insertion into the host chromosome, and preferably also expression, of a CLCNKB encoding sequence included in the rDNA molecule.

Expression control elements that are used for regulating the expression of an operably linked protein encoding sequence are known in the art and include, but are not limited to, inducible promoters, constitutive promoters, secretion signals, enhancers, transcription terminators and other regulatory elements. Preferably, an inducible promoter that is readily controlled, such as being responsive to a nutrient in the host cell's medium, is used.

In one embodiment, the vector containing a CLCNKB encoding nucleic acid molecule will include a prokaryotic replicon, *i.e.*, a DNA sequence having the ability to direct autonomous replication and maintenance of the recombinant DNA molecule intrachromosomally in a prokaryotic host cell, such as a bacterial host cell, transformed therewith. Such replicons are well known in the art. In addition, vectors that include a prokaryotic replicon may also include a gene whose expression confers a detectable marker such as a drug resistance. Typical bacterial drug resistance genes are those that confer resistance to ampicillin (Amp) or tetracycline (Tet).

Vectors that include a prokaryotic replicon can further include a prokaryotic or viral promoter capable of directing the expression (transcription and translation) of the CLCNKB encoding gene sequence in a bacterial host cell, such as *E. coli*. A promoter is an expression control element formed by a DNA sequence that permits binding of RNA polymerase and transcription to occur. Promoter sequences compatible with bacterial hosts are typically provided in plasmid vectors containing convenient restriction sites for insertion of a DNA segment of the present invention. Typical of such vector plasmids are pUC8, pUC9, pBR322 and pBR329 available from Biorad Laboratories (Richmond, CA), pPL and pKK223 available from Pharmacia, Piscataway, NJ.

Expression vectors compatible with eukaryotic cells, preferably those compatible with vertebrate cells, can also be used to express variant rDNA molecules that contain a CLCNKB encoding sequence. Eukaryotic cell expression vectors are well known in the art and are available from several commercial sources. Typically, such vectors provide convenient restriction sites for insertion of the desired DNA segment. Typical of such vectors are pSVL

and pKSV-10 (Pharmacia), pBPV-1/pML2d (International Biotechnologies, Inc.), pTDT1 (ATCC, #31255), the vector pCDM8 described herein, and other like eukaryotic expression vectors.

Eukaryotic cell expression vectors used to construct the rDNA molecules of the present invention may further include a selectable marker that is effective in an eukaryotic cell, preferably a drug resistance selection marker. A preferred drug resistance marker is the gene whose expression results in neomycin resistance, *i.e.*, the neomycin phosphotransferase (*neo*) gene. Southern *et al.*, *J. Mol. Anal. Genet.* (1982) 1:327-341. Alternatively, the selectable marker can be present on a separate plasmid, and the two vectors are introduced by cotransfection of the host cell, and selected by culturing in the presence of the appropriate drug for the selectable marker.

G. Preparation of Host Cells Containing an Exogenously Supplied CLCNKB Encoding Nucleic Acid Molecule

The present invention further provides host cells transformed with a nucleic acid molecule that encodes a human wild-type or altered CLCNKB protein of the present invention. The host cell can be either prokaryotic or eukaryotic. Eukaryotic cells useful for expression of a CLCNKB protein are not limited, so long as the cell line is compatible with cell culture methods and compatible with the propagation of the expression vector and expression of the *CLCNKB* gene product. Preferred eukaryotic host cells include, but are not limited to, yeast, insect and mammalian cells, preferably vertebrate cells such as those from a mouse, rat, monkey or human fibroblastic cell line, the most preferred being cells that do not naturally express a human CLCNKB protein.

Any prokaryotic host can be used to express a CLCNKB-encoding rDNA molecule. The preferred prokaryotic host is *E. coli*.

Transformation of appropriate cell hosts with an rDNA molecule of the present invention is accomplished by well known methods that typically depend on the type of vector and host system employed. With regard to transformation of prokaryotic host cells, electroporation and salt treatment methods are typically employed, see, for example, Cohen *et al.*, *Proc Natl Acad Sci USA* (1972) 69:2110; Maniatis *et al.*, Molecular Cloning, A Laboratory

Manual, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY (1982); Sambrook *et al.*, (1989). With regard to transformation of vertebrate cells with vectors containing rDNAs, electroporation, cationic lipid or salt treatment methods are typically employed, see, for example, Graham *et al.*, *Virology* (1973) 52:456; Wigler *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* (1979) 76:1373-76.

Successfully transformed cells, *i.e.*, cells that contain an rDNA molecule of the present invention, can be identified by well known techniques. For example, cells resulting from the introduction of an rDNA of the present invention can be cloned to produce single colonies. Cells from those colonies can be harvested, lysed and their DNA content examined for the presence of the rDNA using a method such as that described by Southern, *J. Mol. Biol.* (1975) 98:503, or Berent *et al.*, *Biotech.* (1985) 3:208 or the proteins produced from the cell assayed via an immunological method.

H. Production of an CLCNKB Protein Using a Recombinant Nucleic Acid Molecule

The present invention further provides methods for producing a human wild-type or altered CLCNKB protein that uses one of the CLCNKB encoding nucleic acid molecules herein described. Such nucleotides can be used as diagnostic probes, in the production of CLCNKB protein used for screening studies, and for research purposes. In general terms, the production of a recombinant human wild-type or altered CLCNKB protein typically involves the following steps.

First, a nucleic acid molecule is obtained that encodes a CLCNKB protein. If the CLCNKB encoding sequence is uninterrupted by introns, it is directly suitable for expression in any host. If not, then a spliced variant of the CLCNKB encoding nucleic acid molecule can be generated and used or the intron containing nucleic acid molecule can be used in a compatible eukaryotic expression system.

The CLCNKB encoding nucleic acid molecule is then preferably placed in an operable linkage with suitable control sequences, as described above. The expression unit is used to transform a suitable host and the transformed host is cultured under conditions that allow the production of the CLCNKB protein. Optionally the CLCNKB protein is isolated from the

medium or from the cells; recovery and purification of the protein may not be necessary in some instances where some impurities may be tolerated.

Each of the foregoing steps can be done in a variety of ways. For example, the desired coding sequences may be obtained from genomic fragments and used directly in an appropriate host. The construction of expression vectors that are operable in a variety of hosts is accomplished using an appropriate combination of replicons and control sequences. The control sequences, expression vectors, and transformation methods are dependent on the type of host cell used to express the gene and were discussed in detail earlier. Suitable restriction sites can, if not normally available, be added to the ends of the coding sequence so as to provide an excisable gene to insert into these vectors. A skilled artisan can readily adapt any host/expression system known in the art for use with CLCNKB encoding sequences to produce a CLCNKB protein. Particularly well suited are expression systems that result in the production of lipid vesicles containing the expressed protein. Such lipid containing vesicles are well suited for identifying agonists and antagonists of the CLCNKB protein.

I. Ion Transport

As provided above, alterations in the CLCNKB protein cause pathological conditions that are a result of abnormal ion transport. Accordingly, the wild-type and altered variants of the CLCNKB proteins of the present invention can be used in methods to alter the extra or intracellular concentration of Cl^-. In general, the extra or intracellular concentration of Cl^- can be altered by altering the expression of a CLCNKB protein or the activity of a CLCNKB protein. Altering Cl^- levels may also indirectly lead to changes in the levels of Na^+, Ca^{+2}, Mg^{+2} and/or K^+

There are a number of situations in which it is desirable to alter the extra or intracellular concentration of Na^+, Ca^{+2}, Cl^-, Mg^{+2} and/or K^+. Abnormal extra or intracellular pH leads to water retention, increased blood pressure, chronic respiratory and metabolic acidosis, inflammation, sperm activation/inactivation, hydroencephaly, glaucoma, colitis, *etc.* For example, it may be useful to modulate CLCNKB expression to lower blood pressure in individuals who suffer from high blood pressure, as mutations in CLCNKB can cause low blood pressure in individuals so afflicted.

Hence, a CLCNKB protein or *CLCNKB* gene expression can be used as a target for, or as means to alter extra- or intracellular Na^+, Ca^{+2}, Cl^-, Mg^{+2} and/or K^+ concentration. For example, a *CLCNKB* gene can be introduced and expressed in cells to increase *CLCNKB* expression. Alternatively, a small molecule that modulates the activity of a mutant form or a wild-type form of the protein encoded by *CLCNKB* or the *CLCNKB* gene itself might also regulate CLCNKB protein function. Such agents that modulate CLCNKB activity provide the means for regulating extra- and intra-cellular ion levels.

J. Identification of Agents that Bind to the CLCNKB Protein

Another embodiment of the present invention relates to methods for identifying agents that are agonists or antagonists of the CLCNKB proteins herein described, and particularly the native or wild type protein disclosed herein and its allelic variants. Specifically, agonists and antagonists of a CLCNKB protein can be first identified by the ability of the agent to bind to one of the wild-type or altered variants of the CLCNKB proteins herein described. Agents that bind to a CLCNKB protein can then be tested for their ability to stimulate or block ion transport in a *CLCNKB* expressing cell.

In detail, in one embodiment, a CLCNKB protein is mixed with an agent. After mixing under conditions that allow association of CLCNKB with the agent, the mixture is analyzed to determine if the agent bound the CLCNKB protein and upregulate or downregulate CLCNKB activity, respectively. Agonists and antagonists are identified as being able to bind to a CLCNKB protein and upregulated or downregulated CLCNKB activity, respectively. Alternatively or consecutively, as described below, CLCNKB activity can be directly assessed as a means for identifying antagonists and agonists of CLCNKB activity.

The CLCNKB protein used in the above assay can either be an isolated and fully characterized protein, can be a partially purified protein, can be found in a cell that has been altered to express a CLCNKB protein or can be a fraction of a cell that has been altered to express a CLCNKB protein. Further, the CLCNKB protein can be the entire CLCNKB protein or a specific fragment of the CLCNKB protein. It will be apparent to one of ordinary skill in the art that so long as the CLCNKB protein can be assayed for agent binding, *e.g.*, by a shift in molecular weight or change in cellular ion content, the present assay can be used. More

simply, CLCNKB could be bound to a solid support and washed with potential agents that modulate CLCNKB activity. Determining whether the agent could bind to CLCNKB, would be the first step in determining whether it can also modulate CLCNKB activity. Isolation of agents that modulate CLCNKB may not only permit treatment of Bartter's type III carriers, but also individuals suffering from hypertension, edema, congestive heart failure and conditions involving congestive heart failure, nephrotic syndrome, and other diseases involving abnormal fluid volume, because CLCNKB mutations and deletions of the gene itself have been associated with salt wasting and lowered blood pressure.

The methods used to identify whether an agent binds to a CLCNKB protein will be based primarily on the nature of the CLCNKB protein used. For example, a gel retardation assay can be used to determine whether an agent binds to a soluble fragment of a CLCNKB protein whereas patch clamping, voltage clamping, ion-sensitive microprobes or ion-sensitive chromaphores can be used to determine whether an agent binds to a cell expressing a CLCNKB protein and affects the activity of the expressed protein. A skilled artisan can readily employ numerous techniques for determining whether a particular agent binds to a CLCNKB protein.

Once binding is demonstrated, the agent can be further tested, for example, for the ability to modulate the activity of a wild-type or altered variant of the CLCNKB protein using a cell or oocyte expression system and an assay that detects CLCNKB activity. For example, voltage or patch clamping, ion-sensitive microprobes or ion-sensitive chromaphores and expression in *Xenopus* oocytes or recombinant host cells can be used to determine whether an agent that binds a CLCNKB protein can agonize or antagonize CLCNKB activity.

As used herein, an agent is said to antagonize CLCNKB activity when the agent reduces CLCNKB activity otherwise present. The preferred antagonist will selectively antagonize CLCNKB, not affecting any other cellular proteins, particularly other ion transport proteins (*e.g.*, ATP -sensitive K^+ channel ROMK). Further, the preferred antagonist will reduce CLCNKB activity by more than about 50%, more preferably by more than about 90%, most preferably substantially eliminating all CLCNKB activity.

As used herein, an agent is said to agonize CLCNKB activity when the agent increases CLCNKB activity otherwise present. The preferred agonist will selectively agonize normal,

altered or mutant variants of CLCNKB, not affecting any other cellular proteins, particularly other ion transport proteins. Further, the preferred agonist will increase CLCNKB activity by more than about 50%, more preferably by more than about 90%, most preferably more than doubling the level of CLCNKB activity, without, for example, affecting TSC, NKCC2 or ROMK ion transport proteins.

Agents that are assayed in the above method can be randomly selected or rationally selected or designed. As used herein, an agent is said to be randomly selected when the agent is chosen randomly without considering the specific sequences of the CLCNKB protein. An example of randomly selected agents is the use of a chemical library or a peptide combinatorial library, or a growth broth of an organism.

As used herein, an agent is said to be rationally selected or designed when the agent is chosen on a non-random basis which takes into account the sequence of the target site and/or its conformation in connection with the agent's action. Agents can be rationally selected or rationally designed by utilizing the peptide sequences that make up the CLCNKB protein. For example, a rationally selected peptide agent can be a peptide whose amino acid sequence is identical to a fragment of a CLCNKB protein.

The agents of the present invention can be, as examples, peptides, small molecules, and vitamin derivatives, antisense nucleic acid sequences that hybridize (under conditions of stringency appropriate to produce a detectable level of specific binding) to CLCNKB encoding mRNA or DNA, antibodies that bind CLCNKB, as well as carbohydrates. A skilled artisan can readily recognize that there is no limit as to the structural nature of the agents of the present invention. One class of agents of the present invention are peptide agents whose amino acid sequences are chosen based on the amino acid sequence of the CLCNKB protein.

The peptide agents of the invention can be prepared using standard solid phase (or solution phase) peptide synthesis methods, as is known in the art. In addition, the DNA encoding these peptides may be synthesized using commercially available oligonucleotide synthesis instrumentation and produced recombinantly using standard recombinant production systems. The production using solid phase peptide synthesis is necessitated if non-gene-encoded amino acids are to be included.

Another class of agents of the present invention are antibodies immunoreactive with critical positions of the CLCNKB protein. As described above, antibodies are obtained by immunization of suitable mammalian subjects with peptides, containing as antigenic regions, those portions of the CLCNKB protein intended to be targeted by the antibodies. These antibodies can be polyclonal or monoclonal, chimeric antibodies, human antibodies or humanized antibodies.

K. Uses of Agents that Bind to an CLCNKB Protein

As provided in the Background section, the CLCNKB proteins are involved in regulating intracellular and extracellular ion concentration with respect to the renal chloride channel. Agents that bind a CLCNKB protein and act as an agonist or antagonist can be used to modulate biological and pathologic processes associated with CLCNKB function and activity. In detail, a biological or pathological process mediated by CLCNKB can be modulated by administering to a subject in a therapeutically effective dose an agent that binds to a CLCNKB protein and acts as an agonist or antagonist of CLCNKB activity.

As used herein, a subject can be any mammal, so long as the mammal is in need of modulation of a pathological or biological process mediated by CLCNKB. The term "mammal" means an individual belonging to the class *Mammalia*. The invention is particularly useful in the treatment of human subjects.

As used herein, a biological or pathological processed mediated by CLCNKB refers to the variety of cellular events mediated by a CLCNKB protein. Pathological processes refer to a category of biological processes which produce either a deleterious effect or a beneficial effect. For example, pathological processes mediated by CLCNKB are characterized as resulting in a phenotype that exhibits hypokalemic alkalosis with salt wasting, low blood pressure, normal magnesium and hyper- or normocalciuria. These pathological processes can be modulated using agents that reduce or increase the activity of a CLCNKB protein. Preferably, the agent will act to antagonize the activity of normal CLCNKB proteins for the treatment of hypertension such as essential hypertension and congestive heart failure and conditions related to congestive heart failure. Also contemplated are agents that modulate the activity of an otherwise suboptimally active mutant or variant of a CLCNKB protein, for

example to increase the activity of a mutant or, variant characterized by subnormal levels of activity.

As used herein, an agent is said to modulate a pathological process when the agent reduces the degree or severity of the process. For example, an agent is said to modulate blood pressure in a patient with a normal CLCNKB gene when the patient's blood pressure is increased or decreased relative to his or her blood pressure if left untreated with the agent, including otherwise normal intra-and extracellular ion concentrations.

L. Administration of Agonists and Antagonists of an CLCNKB Protein

Agonists and antagonists of the CLCNKB protein can be administered via parenteral, subcutaneous, intravenous, intramuscular, intraperitoneal, transdermal, or buccal routes. Alternatively, or concurrently, administration may be by the oral route. The dosage administered will be dependent upon the age, health, and weight of the recipient, kind of concurrent treatment, if any, frequency of treatment, and the nature of the effect desired. For example, to treat pathological conditions resulting from abnormal ion transport, such as water retention, increased blood pressure, chronic respiratory and metabolic acidosis, inflammation, *etc.*, an agent that modulates native CLCNKB activity is administered systemically or locally to the individual being treated. As described below, there are many methods that can readily be adapted by the skilled artisan to administer such agents.

The present invention further provides compositions containing an antagonist or agonist of a CLCNKB protein that is identified by the methods herein described. While individual needs vary, a determination of optimal ranges of effective amounts of each component is within the skill of the art. Typical dosages comprise from about 0.1 to about 100 g/kg body weight. The preferred dosages comprise from about 0.1 to about 10 g/kg body weight. The most preferred dosages comprise from about 0.1 to about 1 g/kg body weight.

In addition to the pharmacologically active agent, the compositions of the present invention may contain suitable pharmaceutically acceptable carriers comprising excipients and auxiliaries which facilitate processing of the active compounds into preparations which can be used pharmaceutically for delivery to the site of action. Suitable formulations for parenteral administration include aqueous solutions of the active compounds in water-soluble variant, for

example, water-soluble salts. In addition, suspensions of the active compounds and as appropriate, oily injection suspensions may be administered. Suitable lipophilic solvents or vehicles include fatty oils, for example, sesame oil, or synthetic fatty acid esters, for example, ethyl oleate or triglycerides. Aqueous injection suspensions may contain substances which increase the viscosity of the suspension and include, for example, sodium carboxymethyl cellulose, sorbitol, and/or dintran. Optionally, the suspension may also contain stabilizers. Liposomes can also be used to encapsulate the agent for delivery into the cell.

The pharmaceutical formulation for systemic administration according to the invention may be formulated for enteral, parenteral or topical administration. Indeed, all three types of formulations may be used simultaneously to achieve systemic administration of the active ingredient. Suitable formulations for oral administration include hard or soft gelatin capsules, pills, tablets, including coated tablets, elixirs, suspensions, syrups or inhalations and controlled release variants thereof.

M. Combination Therapy

The agents of the present invention that modulate CLCNKB activity can be provided alone, or in combination with another agents that modulate a particular biological or pathological process. For example, an agent of the present invention that reduces CLCNKB activity can be administered in combination with other agents that affect the target ion transporter or related transporters, for example, a diuretic agent. As used herein, two agents are said to be administered in combination when the two agents are administered simultaneously or are administered independently in a fashion such that the agents will act at the same time.

N. Animal Models and Gene Therapy

The *CLCNKB* gene and the CLCNKB protein can also serve as targets for gene therapy in a variety of contexts. For example, in one application, CLCNKB-deficient non-human animals can be generated using standard knock-out procedures to inactivate the *CLCNKB* gene. Alternatively, if such animals are non-viable, inducible CLCNKB antisense molecules can be used to regulate CLCNKB activity/expression. An animal can be altered so as to contain a mutant *CLCNKB* or antisense-CLCNKB expression unit that directs the expression of

CLCNKB or the antisense molecule in a tissue specific fashion. In such uses, a non-human mammal, for example a mouse or a rat, is generated in which the expression of a normal or mutant *CLCNKB* gene is altered by inactivation or activation. This can be accomplished using a variety of art-known procedures such as targeted recombination. Once generated, the CLCNKB-deficient animal, the animal that expresses CLCNKB in a tissue specific manner, or an animal that expresses an antisense molecule can be used to (1) identify biological and pathological processes mediated by CLCNKB; (2) identify proteins and other genes that interact with CLCNKB; (3) identify agents that can be exogenously supplied to overcome CLCNKB deficiency; and (4) serve as an appropriate screen for identifying mutations within *CLCNKB* that increase or decrease activity.

For example, it is possible to generate transgenic mice expressing the human minigene for CLCNKB in a tissue specific-fashion and test the effect of over-expression of the protein in cells and tissues that normally do not contain CLCNKB. This strategy has been successfully used for other proteins, namely *bcl-2* (Veis *et al.*, (1993) *Cell* 75:229). Such an approach can readily be applied to the CLCNKB protein and can be used to address the issue of a potential beneficial effect of CLCNKB in a specific tissue area.

In another embodiment, genetic therapy can be used as a means for modulating a CLCNKB-mediated biological or pathological processes. For example, it may be desirable to introduce into a subject being treated a genetic expression unit that encodes a modulator of CLCNKB expression, such as an antisense encoding nucleic acid molecule or a CLCNKB encoding nucleic acid molecule, or a functional CLCNKB expression unit. Such modulators can either be constitutively produced or inducible within a cell or specific target tissue. This allows a continual or inducible supply of a modulator of CLCNKB or the protein expression within a subject.

O. Antisense Therapy

Another aspect of the invention relates to the use of the isolated nucleic acids in "antisense" therapy. As used herein, "antisense therapy" refers to administration or *in situ* generation of oligonucleotides or their derivatives which specifically hybridize (*e.g.*, bind) under cellular conditions, with the cellular mRNA and/or genomic DNA encoding CLCNKB

so as to inhibit expression of the encoded protein, *e.g.*, by inhibiting transcription and/or translation. The binding may be by conventional base pair complimentarity, or, for example, in the case of binding to DNA duplexes, through specific interactions in the major groove of the double helix. In general, "antisense therapy" refers to the range of techniques generally employed in the art, and includes any therapy which relies on specific binding to oligonucleotide sequences.

An antisense construct of the present invention can be delivered, for example, as an expression plasmid which, when transcribed in the cell, produces RNA which is complementary to at least a unique portion of the cellular mRNA which encodes a CLCNKB protein. Alternatively, the antisense construct is an oligonucleotide probe which is generated *ex vivo* and which, when introduced into the cell causes inhibition of expression by hybridizing with the mRNA and/or genomic sequences of a *CLCNKB* gene. Such oligonucleotide probes are preferably modified oligonucleotides which are resistant to endogenous nucleases, *e.g.*, exonucleases and/or endonucleases, and is therefore stable *in vivo*. Exemplary nucleic acid molecules for use as antisense oligonucleotides are phosphoramidate, phosphothioate and methylphosphonate analogs of DNA (see also U.S. Patent Nos. 5,176,996; 5,264,564; and 5,256,775). Additionally, general approaches to constructing oligomers useful in antisense therapy have been reviewed, for example, by Van der Krol *et al.*, (1988) *Biotechniques* 6: 958-976; and Stein *et al.*, (1988) *Cancer Res.* 48: 2659-2668.

Accordingly, the modified oligomers of the invention are useful in therapeutic, diagnostic, and research contexts. In therapeutic applications, the oligomers are utilized in a manner appropriate for antisense therapy in general. For such therapy, the oligomers of the invention can be formulated for a variety of routes of administration, including systemic and topical or localized administration. For systemic administration, injection is preferred, including intramuscular, intravenous. intraperitoneal, and subcutaneous. For injection, the oligomers of the invention can be formulated in liquid solutions, preferably in physiologically compatible buffers such as Hank's solution or Ringer's solution. In addition, the oligomers may be formulated in solid form and redissolved or suspended immediately prior to use. Lyophilized forms are also contemplated.

The compounds can be administered orally, or by transmucosal or transdermal means. For transmucosal or transdermal administration, penetrants appropriate to the barrier to be permeated are used in the formulation. Such penetrants are known in the art, and include, for example, for transmucosal administration, bile salts and fusidic acid derivatives, and detergents. Transmucosal administration may be through nasal sprays or using suppositories. For oral administration, the oligomers are formulated into conventional oral administration forms such as capsules, tablets, and tonics. For topical administration, the oligomers of the invention are formulated into ointments, salves, gels, or creams as known in the art.

In addition to use in therapy, the oligomers of the invention may be used as diagnostic reagents to detect the presence or absence of the target DNA or RNA sequences to which they specifically bind.

The antisense constructs of the present invention, by antagonizing the normal biological activity of CLCNKB, can be used in the manipulation of ion concentration, both *in vivo* and in *ex vivo* tissue cultures. This aspect of invention is particularly contemplated for use in individuals suffering from hypertension, high blood pressure, nephrotic syndrome, other chronic conditions involving abnormal fluid volume and the like.

The following examples are intended to illustrate, but not to limit, the various aspects of the present invention.

Examples

EXAMPLE 1

Identification and characterization of CLCNKA and CLCNKB

DNA samples were collected from 66 kindreds Bartter's syndrome kindreds, defined on the basis of spontaneous hypokalemic metabolic alkalosis with hyper or normocalciuria and normal magnesium levels. Kindreds were recruited via ascertainment of affected index cases. Genomic DNA was prepared from venous blood of members of Bartter's syndrome kindreds by standard procedures (Bell, G., et al., Proc. Natl Acad. Sci. U.S.A. 78:5759-5763 (1981)). Screening of coding exons of NKCC2 and ROMK for mutation by single strand conformational polymorphism (SSCP) has identified 40 mutant

alleles in 22 of these kindreds (Unpublished data; Bettinelli, A., *et al., J. Pediatr.* 120:38-43 (1992); Simon, D.B., *et al., Nature Genet.* 13: 183-188 (1996)) but has failed to identify mutations in these genes in the remaining kindreds (Orita, M. *et al., PROC. NATL ACAD. SCI. U.S.A.* 86:2766-2770 (1989)). These observations suggest that mutation in an additional gene or genes could account for the disease in these families.

Genomic organization of CLCNKA and CLCNKB.

PAC and cosmid clones containing the human genomic CLCNKA and CLCNKB genes were identified by hybridization to a fragment of CLCNKA cDNA. Maps and intron-exon organization of each gene were determined as described previously. The size of introns of each gene is either known precisely from sequence data or estimated from the size of products specifically derived from each gene by PCR using primers in adjacent exons. The distance between the two genes was determined by long-range PCR using primers specific for the 3' end of the A gene and the 5' end of the B gene (primers hCLCA8: TCAGTCCCTCTTCGTGACATC and hCLCB7: CTCGGACCACACTCTCAACAG). All DNA sequence analysis was by the dideoxy chain termination method using an ABI 377 instrument following a standard protocol.

To ensure that these findings do not simply reflect incomplete mutation detection, markers tightly linked to these two loci were genotyped in 11 kindreds in which affected subjects were the offspring of consanguineous union in order to test for linkage by homozygosity (Lander, E. S. , et al., Science 236: 1567-1570 (1987). One of 11 index cases showed homozygosity for markers spanning the locus of NKCC2, while none of these 11 showed homozygosity at markers spanning the ROMK locus. These results are not significantly different from the expectation under the null hypothesis of no linkage and indicate that mutations in other unlinked loci account for Bartter's syndrome in the vast majority if not all of these kindreds.

Characterization of the CLCNKA and CLCNKB locus

The chloride channels CLCNKA and CLCNKB were then considered as candidate genes in these families. PAC and cosmid genomic clones encoding these genes were isolated; analysis of the results of both PCR and Southern blotting demonstrated the presence of both genes on all six PAC clones, indicating that these two genes are closely linked to one another, as previously suggested by in situ hybridization using CLCNK cDNA (Saito-Ohara, F., *et al.*,(1996) *Genomics* 36:372-374).

The intron-exon organization of each gene was determined, revealing that the two channel genes have identical organization, with each channel encoded in 19 exons (**Figure 1A**). The lengths of the introns of each gene are generally similar. Overall, the genes show 94% DNA sequence identity in exons.

The topographic relationship of these two genes in genomic DNA was determined by performing PCR. Use of primers extending 3' from the 3' end of CLCNKA and 5' from the 5' end of CLCNKB resulted in a specific product whose sequence contained the bona fide 3' end of CLCNKA linked to the 5' end of CLCNKB, indicating that the two genes are transcribed from the same DNA strand, with CLCNKA 5' of CLCNKB (**Figure 1B**). From the size of the product, it was inferred that the two genes are separated by 11 kb in genomic DNA. This organization is independently confirmed by the structure of a chromosome bearing a deletion in kindred BAR226.

SSCP, DNA sequencing and Southern Blotting

Molecular variants in genes were sought using SSCP as previously described (Orita, M., *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* 86:2766-2770 (1989)). All primer pairs used for single exons generate products with sizes between 150 and 300 base pairs (Figure 9). Identified variants were eluted from gel, reamplified by PCR, and DNA from both strands was sequenced in all cases. In the case of deleted exons, control amplifications were performed in each PCR reaction, and all deleted exons were confirmed by at least two independent amplifications.

Comparison of the ratio of amplification of exons of CLCNKA:CLCNKB was performed by analysis of the products of PCR resulting from 30 cycles of amplification

employing primers that amplify exon 7 of both genes. Products were fractionated by SSCP, and the products quantitated using a Molecular Dynamics Phosphoimager™. The results following 25 and 35 cycles of amplification were also analyzed and were not significantly different.

Southern blotting was performed by digestion of genomic DNA with BglII followed by fractionation of the resulting products by electrophoresis on 0.7% agarose gels, transfer to nylon membranes and hybridization with ^{32}P-labeled probes.

EXAMPLE 2

Genetic Localization of CLCNKA and CLCNKB and Linkage to Bartter's Syndrome

Genotyping of marker loci was performed by polymerase chain reaction using specific primers as described previously (Simon, D.B., *et al.*, (1996) *Nature Genet.* 13:183-188). Markers genotyped to test linkage to NKCC2 were D15S132, D15S161 and D15S209; markers typed to test linkage to ROMK were D11S968, D11S912, and D11S4198. Linkage by homozygosity mapping in kindreds with affected offspring of consanguineous marriage was analyzed from calculation of the likelihood of homozygosity at all tightly linked marker loci under the null hypothesis and the alternative hypothesis of linkage in a fraction of kindreds, specifying heterozygosities of markers D1S2728, D1S436 and D1S2697 of 74%, 75% and 70%, respectively (Gyapay, G., *et al., Nature Genet.* 7:246-339 (1994)). The likelihood of all markers being homozygous under the null hypothesis is the likelihood that the index case has inherited the same chromosome segment on both alleles by descent from an ancestor by chance, plus the likelihood of independent markers being homozygous by state. The LOD score for linkage, allowing for locus heterogeneity, is calculated from a LR(CLCNK) + (1- a) where a is the proportion of families linked to locus CLCNK, and LR(CLCNK) is the likelihood ratio favoring linkage at CLCNK; the LOD score is calculated as the log10 of these values summed over all families (Ott, J. Analysis of Human Genetic Linkage (Johns Hopkins University Press, Baltimore 1985)).

These loci were localized on the human genetic map by analysis of linkage in CEPH kindreds, comparing the inheritance of a triallelic polymorphism in exon 13 of CLCNKA (marker CLCKA13) to inheritance of other loci on chromosome 1. Multipoint analysis of linkage demonstrated a loss of odds (LOD) score of 15.8 for linkage to D1S436 at a recombination fraction of zero, and localized the gene with odds of greater than 1000:1 to a 10 cM segment within the interval flanked by D1S507 and D1S199 (Figure 1C).

These results identified highly polymorphic loci closely linked to CLCNKA and CLCNKB. Three of these loci, D1S436, D1S2697 and D1S2728 (estimated recombination fractions with CLCKA13 of zero, zero and 3 cM) were genotyped in the 11 consanguineous Bartter's Syndrome type III kindreds that showed no evidence of linkage to NKCC2 or ROMK. Affected subjects in five of these kindreds were homozygous at all loci (Figure 2), consistent with linkage in a subset of these families. The LOD score for linkage of Bartter's syndrome and CLCNKA/CLCNKB, allowing for locus heterogeneity, is 4.72 at a recombination fraction of zero, providing significant evidence for linkage.

EXAMPLE 3

Deletions in CLCNKB

These foregoing findings motivated an examination of CLCNKA and CLCNKB for mutations in Bartter's kindreds not showing mutation in NKCC2 or ROMK. All exons were screened for variants in the index case of each kindred by SSCP using primers that either selectively amplify CLCNKA or CLCNKB or are promiscuous, amplifying segments of both of these highly similar genes.

The results demonstrated the absence of amplification of either the entire CLCNKB gene or substantial portions of this gene in affected members of 10 kindreds (Refer to **Figure 3A-E** and Figure 5), consistent with homozygous loss of CLCNKB. For example, the index case of kindred BAR251 (labeled 251-1) shows no amplification using primers that specifically amplify exons 1-2, 6-9 or 17-19 of CLCNKB (**Figures 3A, 3B** and **3C**). Similarly, the product of CLCNKB is absent while the product of

CLCNKA is present when primers that amplify both of these genes are used (**Figures 3D and 3E**); none of the individual exons of CLCNKB can be amplified by PCR (Figure 5). This absence of CLCNKB co-segregates with the disease, as none of six parents of children showing homozygous loss is homozygous for the absence of CLCNKB (**Figure 3E**). The complete loss of CLCNKB is confirmed by Southern blotting of genomic DNA using cDNA probes corresponding to exons 3-9 or 10-17 of CLCNKB (Figure 4). This same pattern of complete loss of CLCNKB is seen in six additional kindreds (Figures 3-5). Absence of CLCNKB exons was not detected in any of 80 unrelated unaffected subjects screened.

The observed loss of CLCNKB could result from two alternative mechanisms-deletion or gene conversion with CLCNKA sequences. These possibilities can be distinguished by comparing the number of CLCNKA and CLCNKB genes in heterozygous carriers of these mutant alleles. In the case of gene deletion, the heterozygotes will have 2 copies of CLCNKA and one copy of CLCNKB; in the case of gene conversion, this ratio will be 3:1. The amplification of exon 7 of CLCNKA and CLCNKB was compared using a primer set that amplifies both genes, in 10 normal controls and six heterozygous parents of patients with homozygous loss of CLCNKB (for example, **Figure 3E**). The results demonstrate a mean ratio of CLCNKA:*CLCNKB* of 1.97:1 in heterozygotes (standard deviation 0.12, range 1.87 to 2.10:1), very close to the 2:1 ratio expected for deletion and inconsistent with the 3:1 ratio expected for gene conversion.

Two kindreds showed homozygous loss of part of CLCNKB. In kindred BAR276 PCR reveals absence of exons 15-19 of CLCNKB (Figures 3 and 5) and Southern blotting demonstrates replacement of the normal 3' BglII fragment with a derivative product which we infer contains exons 10-14 of CLCNKB (**Figure 4A**). Similarly, BAR242 shows absence of exons 3-12 by PCR (Figures 3 and 5); in this case Southern blotting shows absence of the normal 5' BglII product (not shown) and much weaker hybridization to a fragment the size of the normal 3' BglII products (**Figure 4A**), consistent with this being a derivative fragment.

EXAMPLE 4

Deletion of CLCNKB by Unequal Crossover

The tight linkage and topology of CLCNKA and CLCNKB raises the question of whether unequal crossing over between these genes or blocks of homologous flanking sequences could be one mechanism producing deletions of CLCNKB sequences. If unequal crossing over occurred between coding exons of these genes, one would find reciprocal loss of CLCNKA and CLCNKB sequences (Figure 6A(ii)). Just such a pattern is seen in BAR226, with homozygous loss of only exons 1-2 of CLCNKB and homozygous loss of exons 3-19 of CLCNKA (Figures 3-5). If unequal crossing over has fused CLCNKA and CLCNKB sequences, PCR using a 5' primer specific for CLCNKA in exon 2 and a 3' primer specific for CLCNKB in exon 4 should yield a product of 2.0 kb in members of this kindred but not in controls. This expectation is met (**Figure 6A(i)**). Analysis of the DNA sequence of this product confirms that this fragment comprises a molecular chimera of CLCNKA and CLCNKB sequences, with CLCNKA specific sequences extending through exons 1 and 2, then crossing over to CLCNKB-specific sequences in intron 2. Assuming the CLCNKB - specific regulatory sequences lie at or near the 5' end of the gene, we speculate that this sole remaining gene would be regulated as the normal CLCNKA, resulting in loss of a (putative) CLCNKB pattern of expression.

Specific primers in CLCNKA exon 2 (hCLCA13: GCTATGCCATGAACTTTGCCATC) and CLCNKB exon 4 (hCLCB4: AGGAAGAGGGTGCTGCCACAG) were used to amplify a unique chimeric PCR product in the parents and 2 affected members of this kindred.

EXAMPLE 5

Missense and Nonsense Mutations in CLCNKB

Seven different mutations altering the encoded CLCNKB gene product that co-segregate with the disease were also identified (**Figures 6A** and **6B**). Cases in 4 kindreds

were homozygous for identified mutations, two index cases were compound heterozygotes, and a single mutation was identified in one kindred (**Figure 8**). Cases in two apparently unrelated Turkish kindreds, BAR274 and BAR294, are homozygous for the identical mutation, substituting leucine for proline at codon 124 (**Figure 6B(iv)**). This proline residue is conserved among all mammalian members of the CLC family. Two Spanish kindreds, BAR205 and BAR184, are homozygous for substitution of threonine for alanine at codon 204 in the fifth transmembrane domain (**Figure 6B(iii)**). All CLC channel members, including the CLC-related channel of S. cerevesiae , have alanine or glycine at this position, within a highly conserved hydrophobic segment, AAAA or AGAA. Other missense mutations include R438C, where R438 is conserved among all members of the CLC family, A349D, which introduces a charged residue in the eighth transmembrane domain (Figure 6B(i)), and Y432H.

In addition to these missense mutations, there is one nonsense mutation, at codon 513 (**Figure 6B(ii)**), and one splice site mutation at the end of exon 9 mutating the consensus splice site, GT, to TT; loss of this site is expected to result in loss of normal splicing or to result in a frameshift mutation due to aberrant splicing, as the preceding base is G.

None of these mutations was identified on 160 control chromosomes. Moreover, no non-conservative mutations were found in CLCNKA, indicating the specificity of these mutations. In sum, we have identified 11 different mutations altering CLCNKB, accounting for 33 mutant alleles in 17 Bartter's syndrome kindreds.

We have further identified additional Bartter's syndrome type III mutations which are listed in Table I. These mutations were obtained using the methods described above.

TABLE 1

CLCNKB DNA MUTATION	LOCATION AND EFFECT
GT → AT	INTRON 9, 5' END
ATG → GTG	M67 → V
GGC → GAC	G164 → D

CLCNKB DNA MUTATION	LOCATION AND EFFECT
GGG - GGGG	INSERTION OF G AT BASE 15-18 - FRAMESHIFT
CAC - CGC	L27 - R
GTG - GAG	V149 - E
ACCA - ACA	DELETION OF C AT BASE 785 - FRAMESHIFT
12 BP DELETION, BASES 1,192-1,203	DELETION OF I398, F399, G400, T401
ACG - ATG	T562 - M
TGG - TGA	W610 - STOP
TGC - TGA	C667 - STOP
GCC - ACC	A577 - T
GCG - GAG	A242 - E
ATG - ATA	M412 - I
GGG - GAG	G476 - E
CGA - TGA	R595 - STOP
GAG - GGG	E442 - G
TGC - TAC	C626 - Y
ACG - ATG	T562 - M
TGGA - TGA	DELETION OF G AT BASE 1,692 - FRAMESHIFT
GT - AT	INTRON 5, 5' END
TGG - TAG	W391 - STOP

EXAMPLE 6

Physiological and Clinical Features of Patients with CLCNKB Mutations

Examination of the biochemical and clinical features of patients with mutation in CLCNKB reveals that all have spontaneous hypokalemia, with serum K^+ levels as low as 1.1, and all have serum bicarbonate levels that are either frankly elevated or at the upper limits of normal, with some patients having profound elevations in bicarbonate (Figure 8). All have evidence of salt wasting; 12 cases presented in the first year of life with dehydration and life-threatening hypotension. The 5 remaining cases presented with polyuria and/or dehydration at older ages, and all had blood pressures at least one standard deviation below the mean for age and gender. All are dependent on salt supplementation. In six patients studied, all had elevated plasma renin activity and plasma aldosterone concentration, confirming volume depletion. The renal handling of a water load, which permits measurement of fractional chloride reabsorption in the thick ascending limb, was determined in the index cases of BAR218 and BAR226. The fractional reabsorption of chloride in the TAL is normally 80-95%; in these two patients the fractional chloride reabsorption was profoundly depressed at 24% and 25%, respectively, indicating a striking defect in chloride reabsorption in the TAL. Similar results were obtained in three additional index cases. While there is wide variation in urinary calcium:creatinine ratios, eleven of the 17 index cases had hypercalciuria (ratios 0.5-1.7), while six had values in the normal range.

There is marked variation in severity of clinical features of these patients, ranging from near-fatal volume depletion with hypokalemic alkalosis and respiratory arrest (BAR157 and BAR190) or requirement for massive intravenous K^+ replacement (20 meq/kg/day) (BAR276) to very mild disease presenting with only polyuria and weakness at age 16 (BAR184). Even among patients with homozygous deletions, age of presentation and clinical severity is highly variable (Figure 8).

Despite this wide clinical variation, there is one finding that almost completely distinguishes patients with CLCNKB mutations from those with mutations in NKCC2 or ROMK. While nephrocalcinosis (renal calcification which

if untreated leads to renal failure) is almost invariably present in the latter group at birth (diagnosed by renal ultrasound in 19 of 20 index cases studied), this finding is absent among all 17 index cases with mutation in *CLCNKB*. This phenotypic difference between these two groups is highly significant (c^2 1df = 33.1, $p < 0.0001$). Whether this difference is attributable to differences in calcium handling or other physiologic sequelae will require further investigation. For additional material relating to differential diagnosis of Bartter's and Gitelman's syndromes, see Simon, D. et al., Curr. Opin. Nephrol. Hypertens., 7: 43-7 (1998); Simon, D. et al., Curr. Opin. Cell. Biol., 10: 450-454 (1998), and which are herein incorporated by reference in their entirety.

It should be understood that the foregoing discussion and examples present merely present a detailed description of certain preferred embodiments. It therefore should be apparent to those of ordinary skill in the art that various modifications and equivalents can be made without departing from the spirit and scope of the invention.

What is Claimed is:

1. A method of diagnosing a pathological condition associated with a defect in renal ion transport in a subject comprising the steps of:

obtaining a nucleic acid sample from the subject; and

determining whether a *CLCNKB* gene is present or is mutated.

2. The method of claim 1, further comprising the step of comparing the mutation with known mutations characteristic of Bartter's syndrome type III.

3. The method of claim 1, wherein the nature of the mutation contributes to making a differential diagnosis between Bartter's syndrome type III and Gitelman's syndrome, Bartter's syndrome type III and other hypo- or hyper-tensive conditions, or Bartter's syndrome type III and Bartter's syndrome types I and II.

4. The method of claim 1, wherein said nucleic acid sample is analyzed for the presence of a mutation resulting in the absence of a CLCNKB protein, or the presence of a nucleic acid molecule encoding a human CLCNKB protein wherein the CLCNKB protein contains alterations of one or more amino acids when compared to a wild-type human CLCNKB protein, and wherein said alterations are diagnostic of Bartter's syndrome type III (homozygous alteration) or a carrier of Bartter's syndrome type III (heterozygous alteration).

5. The method of claim 1, wherein said method comprises the steps of amplifying nucleic acid molecules in said sample using a nucleic acid amplification method and primers that flank and selectively amplify at least one exon of said *CLCNKB* encoding nucleic acid molecule and determining whether a mutation is present in said amplified nucleic acid molecule.

6. The method of claim 5, wherein said method comprises the steps of amplifying nucleic acid molecules in said sample using a nucleic acid amplification method

and primers that flank and selectively amplify at least one exon of said *CLCNKB* encoding nucleic acid molecule and identifying whether a mutation is present in said amplified nucleic acid molecule.

7. A method to determine the absence of a protein or the presence of a mutated protein conferring altered ion transport, said method comprising the step of analyzing a protein sample for the presence of a mutation that is characteristic of Bartter's syndrome type III.

8. The method of claim 7 wherein the mutant protein is selected from CLCNKB mutations listed in Figure 10C and Table 1.

9. The method of claim 7, wherein said method is used to determine the absence of a protein or the presence of a mutated protein conferring altered ion transport, said method comprising the step of analyzing a protein sample for the absence of or the presence of a mutation in human renal chloride channel protein, CLCNKB.

10. The method of claim 9, wherein said protein sample is analyzed for the presence of a human CLCNKB protein that is altered for one or more amino acids when compared to a wild-type human CLCNKB protein or analyzed for the absence of a human CLCNKB protein.

11. The method of claim 9, wherein said protein sample is analyzed for the presence of a human CLCNKB protein whose amino acid sequence is altered for one or more amino acids from wild-type CLCNKB sequences.

12. The method of claim 9, wherein said protein is analyzed for the presence of an altered human CLCNKB protein by determining whether the altered CLCNKB protein can

bind to an antibody that recognizes and binds to the altered CLCNKB protein and not wild type CLCNKB protein.

13. The method of claim 9, wherein said mutant CLCNKB protein is identified using a method selected from the group consisting of gel-electrophoretic mobility and isoelectric point focusing.

14. An antibody that binds to a mutant human CLCNKB protein but not to a wild-type human CLCNKB protein.

15. An isolated nucleic acid molecule that encodes a human altered variant of the CLCNKB protein or portions thereof.

16. A vector containing a nucleic acid molecule according to claim 15.

17. A host cell transformed to contain a nucleic acid molecule according to claim 15.

18. A method for producing a mutant or altered variant of an CLCNKB protein or portions thereof comprising culturing the host of claim 17 under conditions in which said nucleic acid molecule is expressed to produce a protein encoded thereby.

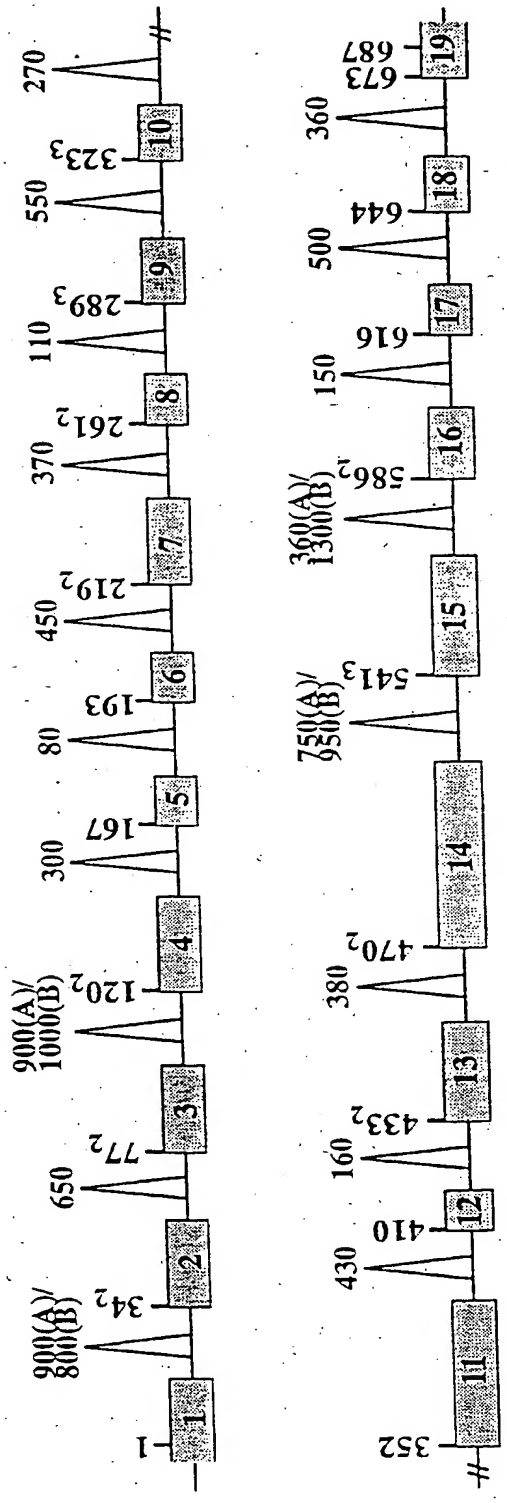
19. A method to identify agents that modulate the activity of the CLCNKB protein, comprising the steps of contacting the protein with said agent under appropriate conditions to permit association between the agent and protein, and determining whether said association results in inhibited or enhanced level of activity of the protein.

20. A composition comprising a pair of PCR primers selected from the primers disclosed in Figure 9 (SEQ ID NOS _____).

21. A test kit for diagnosing Bartter's Syndrome Type III, comprising a pair of PCR primers selected from the primers disclosed in Figure 9 (SEQ ID NOS. _____).

22. A method of treating hypertension, edema, congestive heart failure or conditions as may occur with congestive heart failure, nephrotic syndrome, or conditions involving abnormal fluid volume, comprising the administration of an agent identified by the method of claim 19 in a therapeutically effective dose.

FIG. 1A


1
900(A)/800(B)
34
2
650
77
3
900(A)/1000(B)
120
4
300
167
5
80
193
6
450
219
7
370
261
8
110
289
9
550
323
10
270
#
352
11
430
410
12
160
433
13
380
470
14
750(A)/950(B)
541
15
360(A)/1300(B)
586
16
150
616
17
500
644
18
360
673
19
687

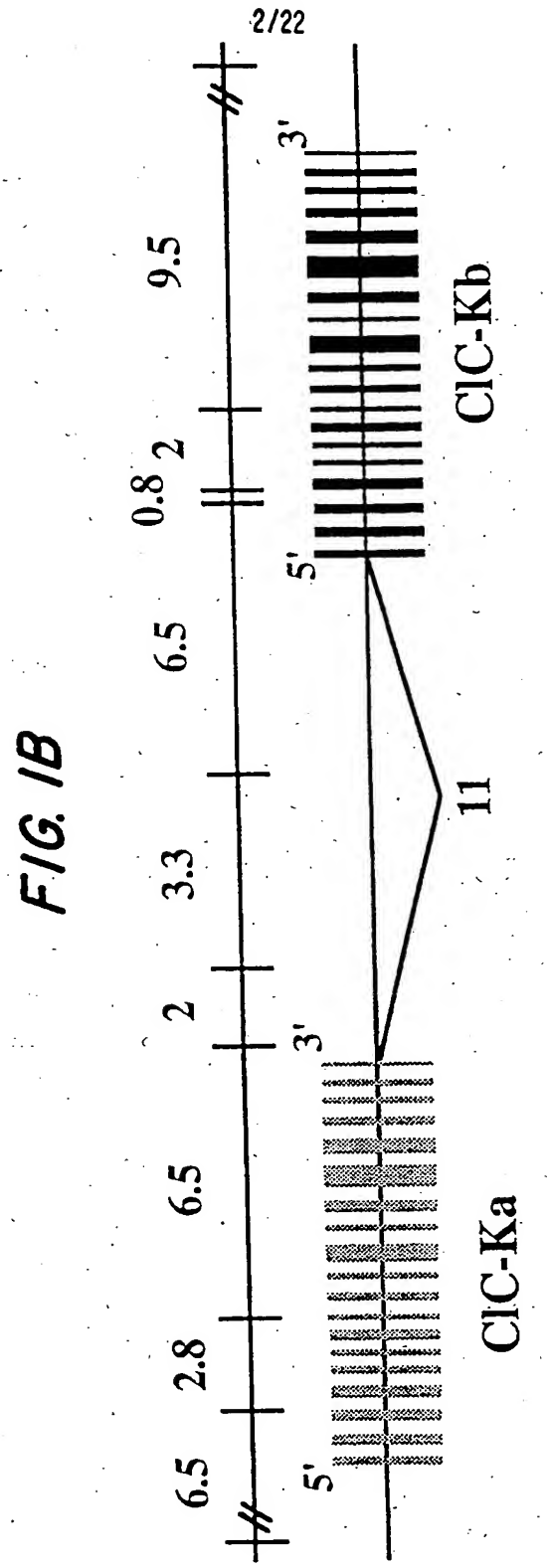
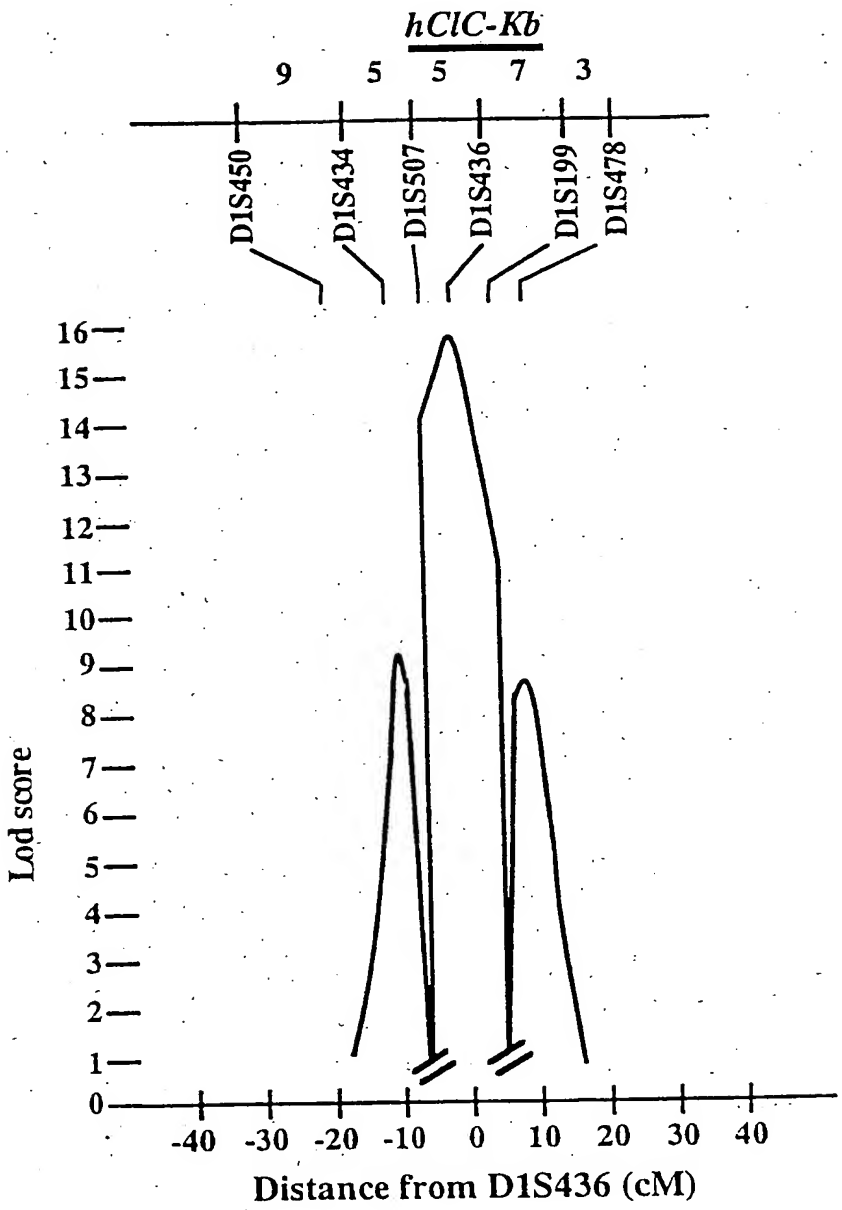


FIG. 1B

FIG. 1C


hClC-Kb
9
5
5
7
3
D1S450
D1S434
D1S507
D1S436
D1S199
D1S478
16
15
14
13
12
11
10
9
8
7
6
5
4
3
2
1
0
Lod score
-40
-30
-20
-10
0
10
20
30
40
Distance from D1S436 (cM)

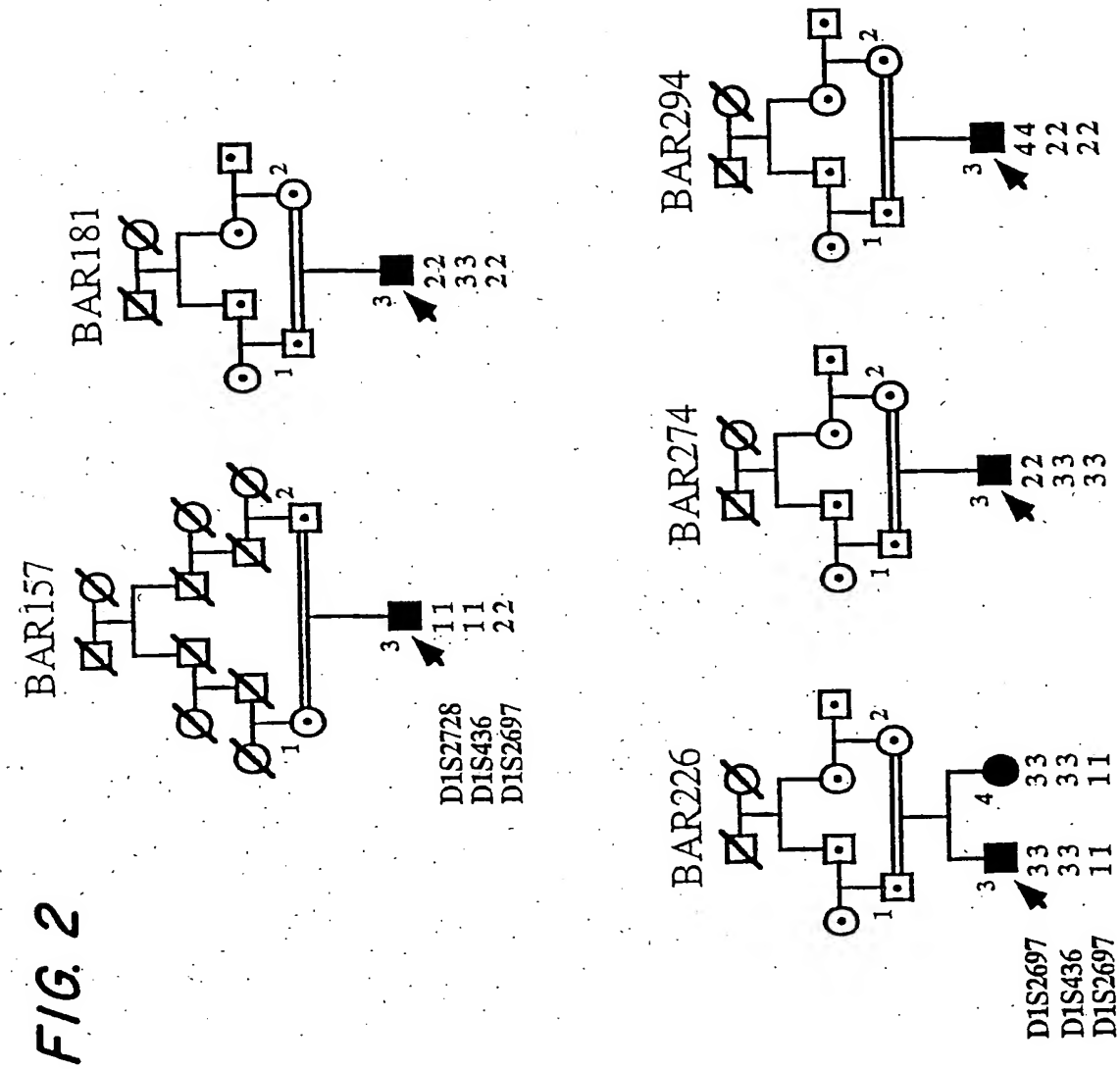

BAR181
22
33
22
BAR157
11
11
22
D1S2728
D1S436
D1S2697
BAR294
44
22
22
BAR274
22
33
33
BAR226
33
33
33
11
D1S2697
D1S436
D1S2697

FIG. 2

FIG. 3A

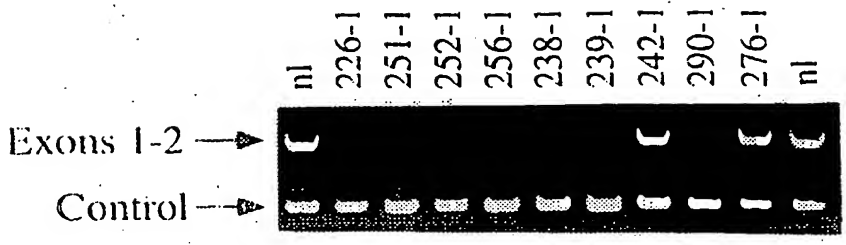


FIG. 3B

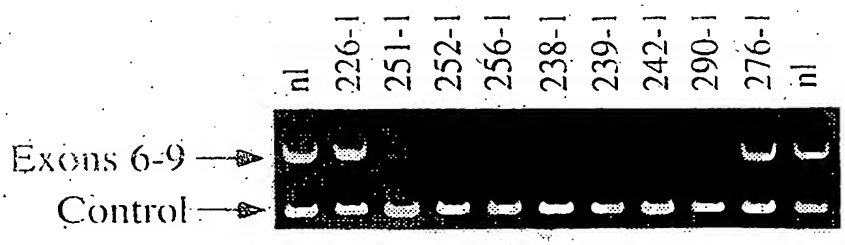


FIG. 3C

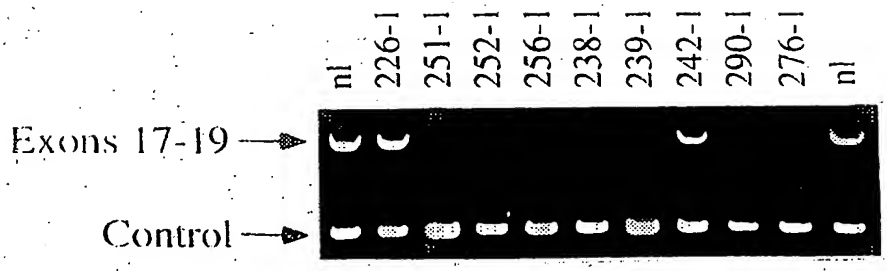
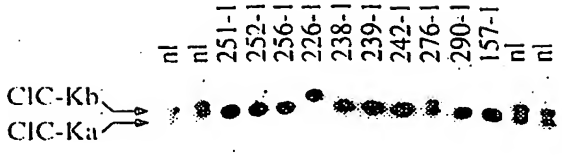


FIG. 3D


nl
nl
251-1
252-1
256-1
226-1
238-1
239-1
242-1
276-1
290-1
157-1
nl
nl
ClC-Kb
ClC-Ka

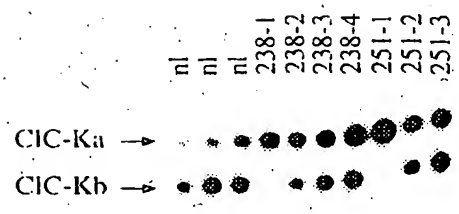
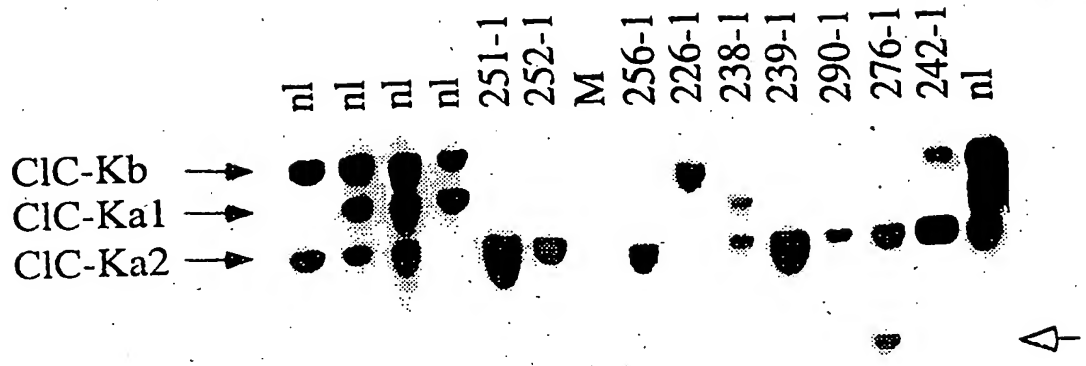

nl
nl
nl
238-1
238-2
238-3
238-4
251-1
251-2
251-3
ClC-Ka
ClC-Kb

FIG. 3E

FIG. 4A


nl
nl
nl
nl
251-1
252-1
M
256-1
226-1
238-1
239-1
290-1
276-1
242-1
nl
ClC-Kb
ClC-Ka1
ClC-Ka2

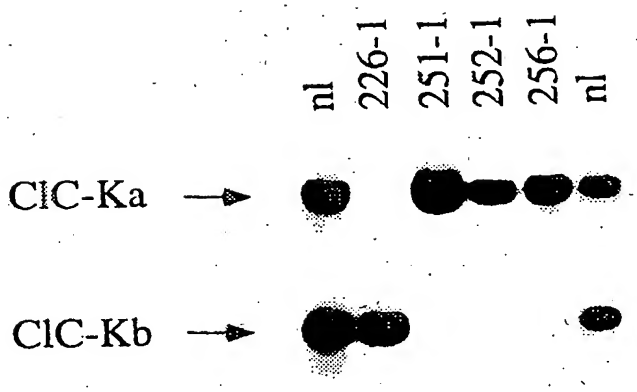

nl
226-1
251-1
252-1
256-1
nl
ClC-Ka
ClC-Kb

FIG. 4B

FIG. 5A

CLCNKA and *CLCNKB* exons deleted in Bartter's syndrome patients

Kindred	*CLCNK*	Exon 1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19
BAR157	A	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	B	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
BAR238	A	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	B	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
BAR239	A	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	B	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
BAR251	A	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	B	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

FIG. 5B

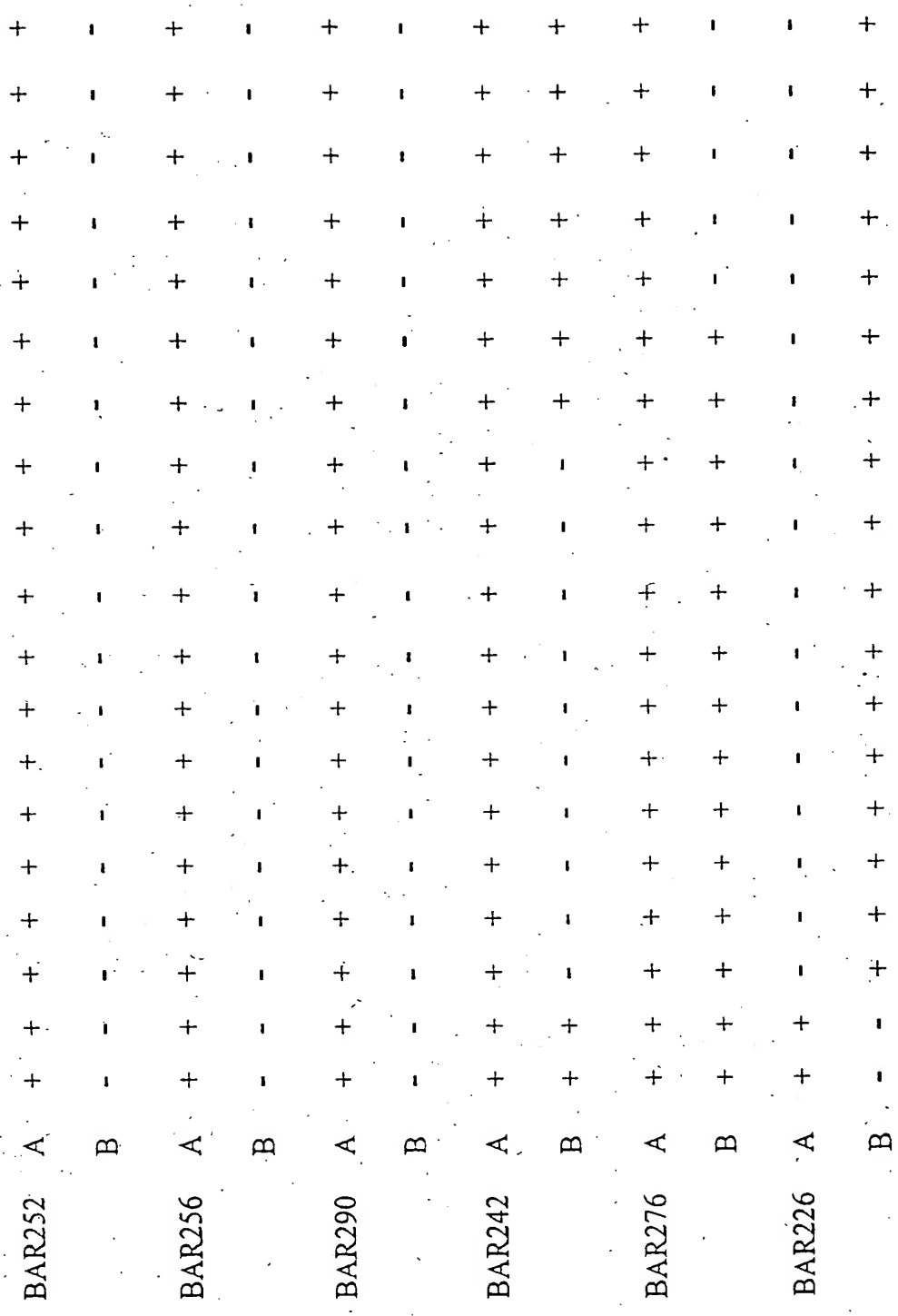


FIG. 6Ai

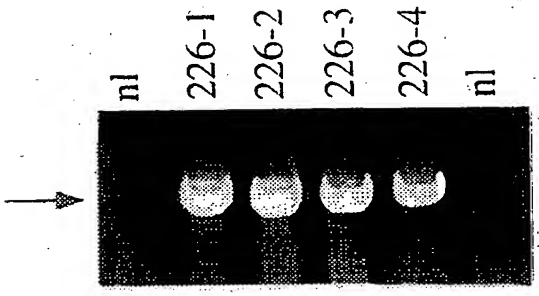


FIG. 6Aii


ClC-Kb
3'
5'
ClC-Ka
3'
5'
Unequal crossing over
ClC-Kb
3'
5'
ClC-Ka
3'
5'
3'
5'
Chimeric ClC-K Gene

FIG. 6Bi

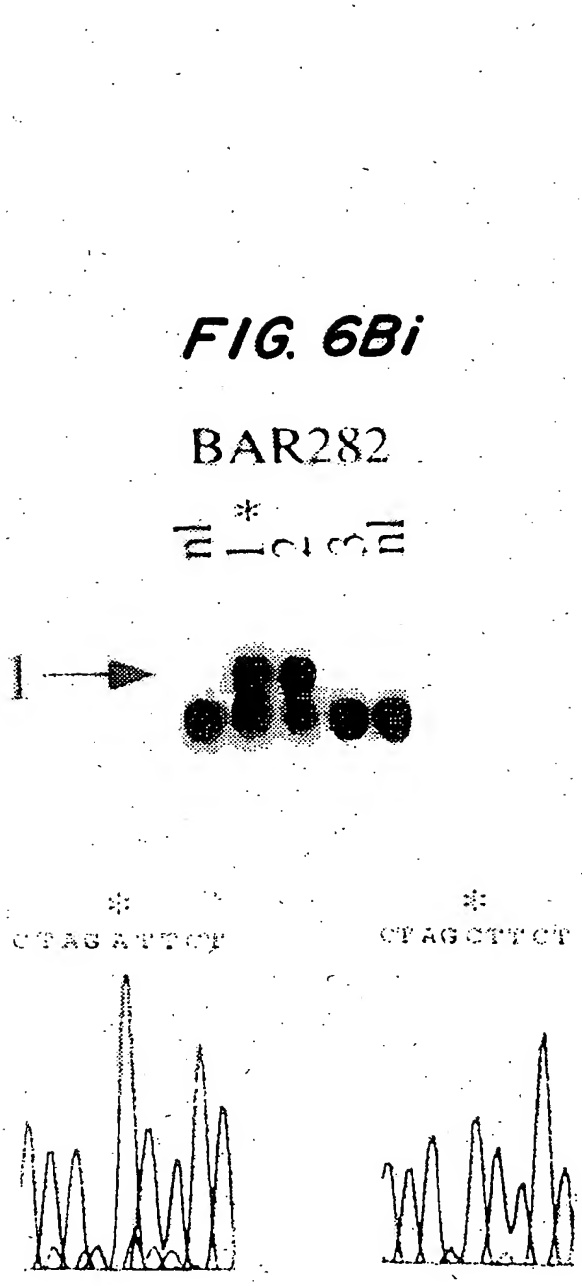


FIG. 6Bii

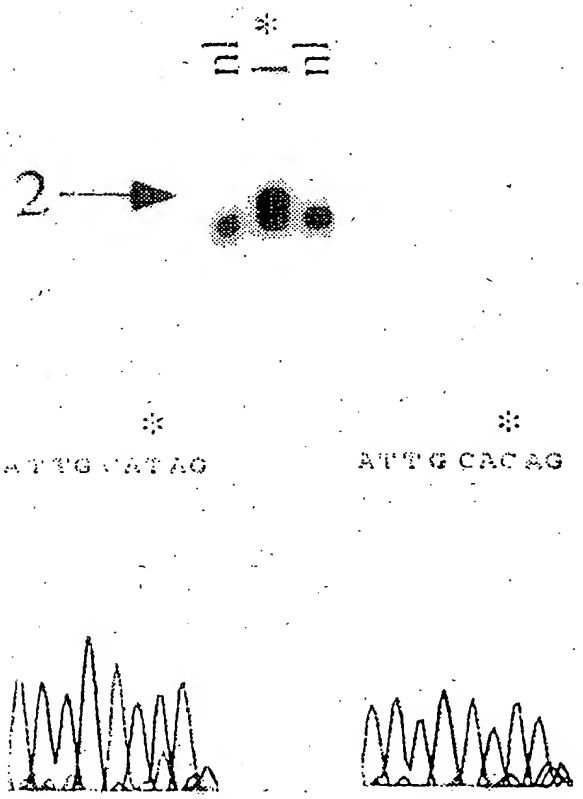


FIG. 6Biii

BAR184, 205

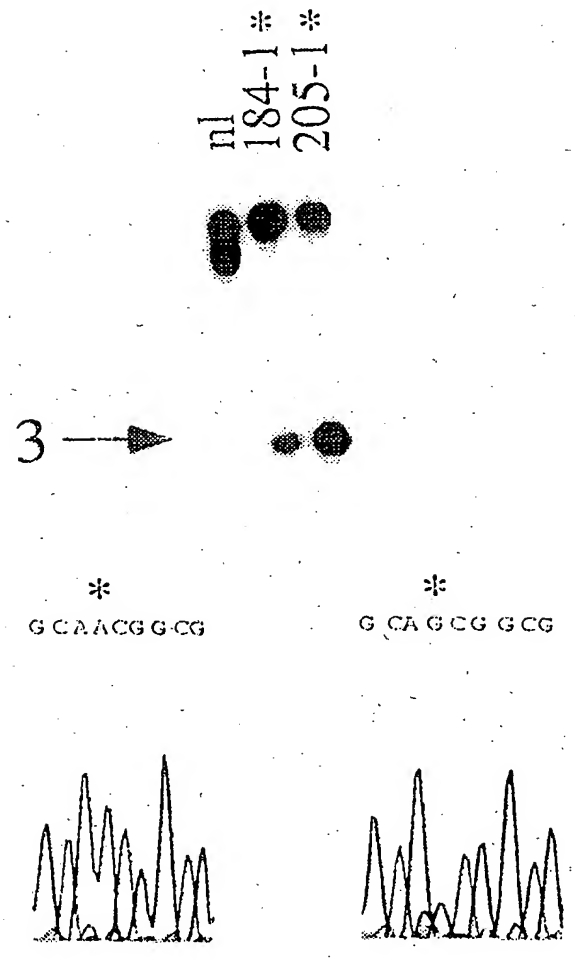


FIG. 6Biv

BAR274, 294

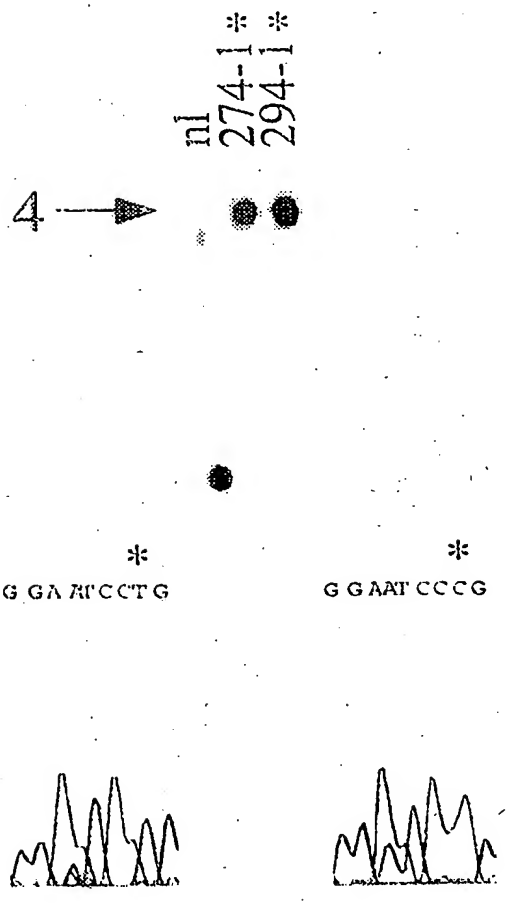


FIG. 7A

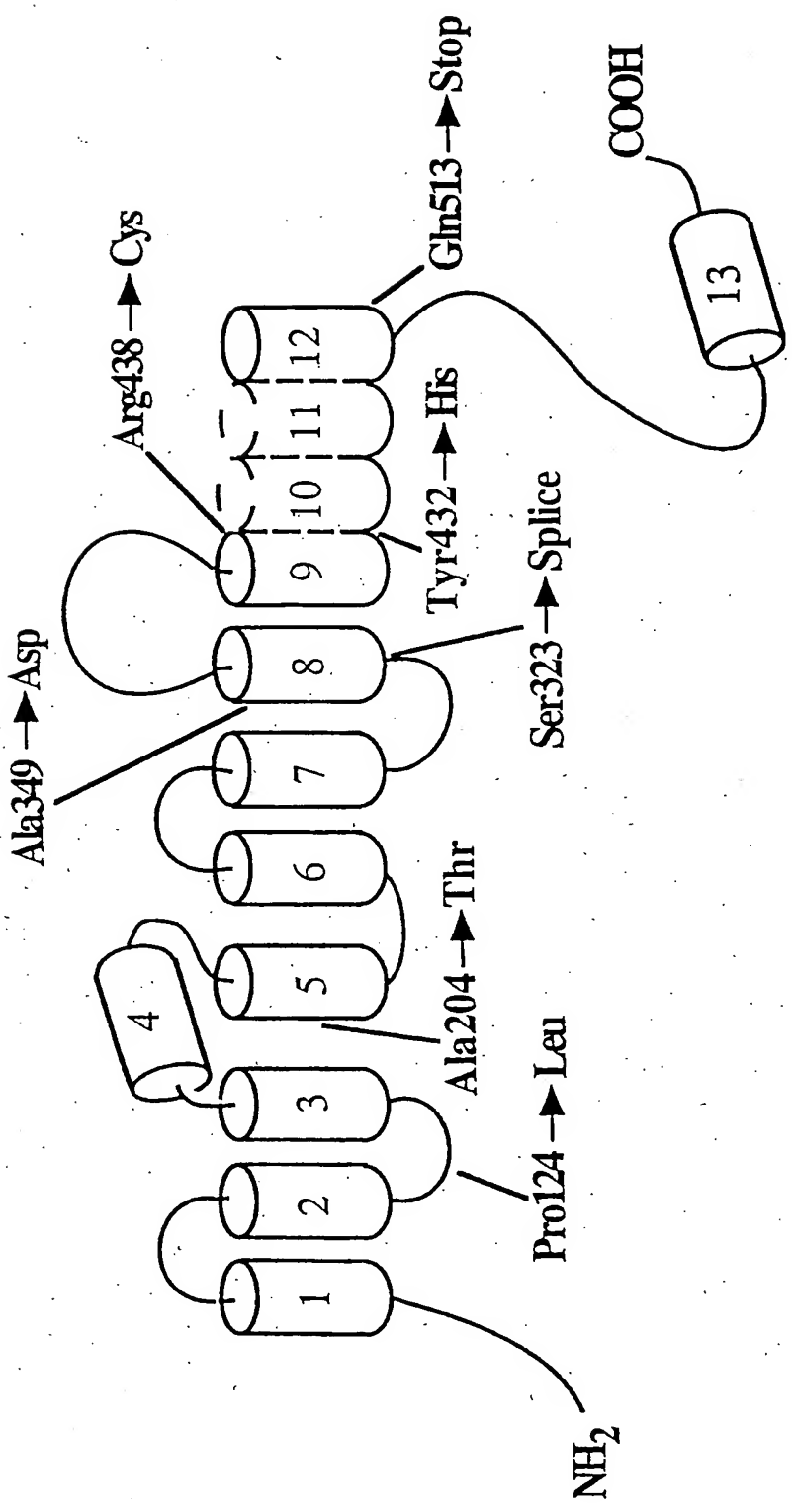

NH2
1
2
3
4
5
6
7
8
9
10
11
12
13
COOH
Pro124 → Leu
Ala204 → Thr
Ala349 → Asp
Arg438 → Cys
Gln513 → Stop
Tyr432 → His
Ser323 → Splice

FIG. 7B

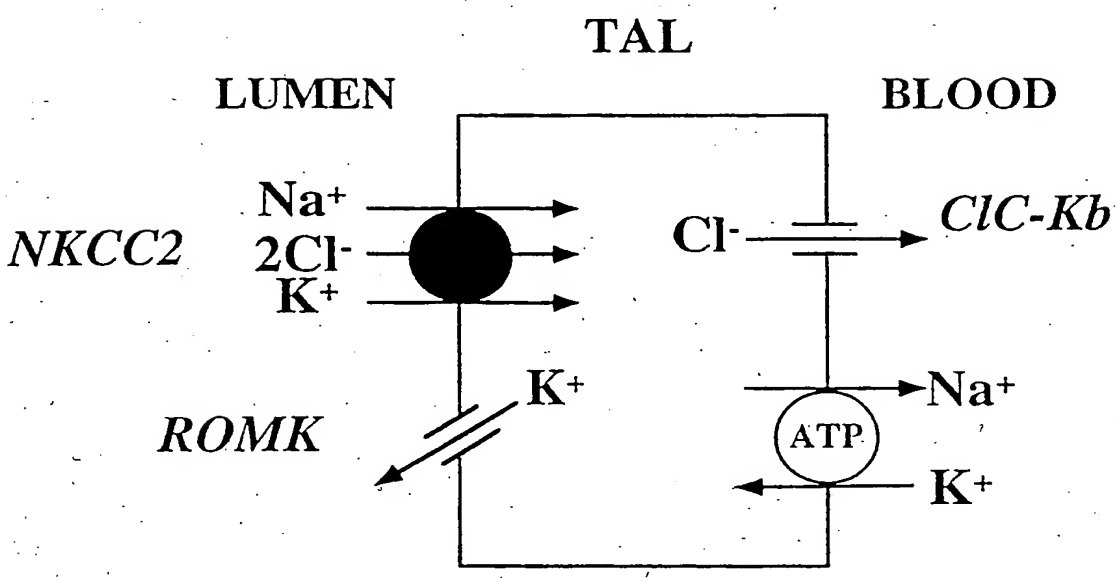


FIG. 8

Clinical characteristics of index cases with Bartter's syndrome

Kindred	Ancestry	Age of Dx	K^+	HCO_3	U_{Cl}/U_{Cr}	Nephrocal.	Mutation
BAR157	Yemen	0.3	1.1	52	1.0	No	
BAR184	Spain	16	2.5	39	0.5	No	
BAR205	Spain	6	2.6	27	1.7	No	A204T*
BAR218	Saudi Arabia	0.7	1.8	35	0.4	No	R438C, Ser323-Splice
BAR226	Saudi Arabia	4	2.5	30	0.2	No	ClC-Kb Crossover*
BAR238	African American	0.3	3.1	29	0.3	No	ClC-Kb Deletion*
BAR239	Portugal	0.1	2.6	28	0.5	No	ClC-Kb Deletion*
BAR242	African American	Birth	2.9	33	0.6	No	ClC-Kb Deletion*
BAR251	Portugal	4	2.3	32	0.8	No	ClC-Kb Deletion*
BAR252	Portugal	0.1	2.7	36	1.7	No	ClC-Kb Deletion*
BAR256	African American	Birth	2.8	34	0.2	No	ClC-Kb Deletion*
BAR274	Turkey	Birth	2.4	33	0.3	No	P124L*
BAR276	African American	0.1	2.1	28	1.5	No	ClC-Kb Deletion*
BAR282	England	Birth	2.4	32	1.4	No	A349D
BAR283	Wales	8	2.9	34	1.1	No	Y432H, Q513Stop
BAR290	African American	Birth	1.6	70	0.3	No	ClC-Kb Deletion*
BAR294	Turkey	1	2.5	29	1.1	No	P124L*

FIG. 9

PCR Primers for Analysis of the Human CLCNKB Gene

Primer Name	Exon	Forward Primer	Reverse Primer
hCLCNKBex1	1	CGGCAGCCACAGCAGGAGGAC	GATGTCCTGAGTGGTCCTCCAG
hCLCNKBex2*	2	TGCCCCACCCTGTGCCGTGAC	CTTGGCCCAGAGCAGCACCTG
hCLCNKBex3	3	GAGGCTGTGGGTGCCTCCCTG	AGTGGGGACTGGCGTAGCGAC
hCLCNKBex4*	4	CCCTCCTGGCCCTGCCCAC	GGGTGGTTGGGATGCCCTCAC
hCLCNKBex5	5	CGTGCTGACTCTGGGTGAGAC	CGAGTCCAGGGCTCAGAGTCAG
hCLCNKBex6	6	GGCTGACTCTGAGCCCTGGAC	GGAGGAGCTTGAGGGACCCAG
hCLCNKBex7	7	GGAGGGCCCACCTGAGATCAG	GCAGGGCCAGGGTCAGGCAG
hCLCNKBex8	8	CGCCATCTTGGCTCCCCACTG	CTCTTCAGGGGCCCAAGGCAC
hCLCNKBex9*	9	GTCAGGCTCTGGGCTCATGTC	AGCTCGCTGAGAGGTCCCCAG
hCLCNKBex10	10	CAGCCCTAGAGCCCACCCATC	GGGCTTCCCCACTCCTGCCAC
hCLCNKBex11	11	CCTCATGTCCAGTTCCCACCTG	GTCCCAGCTCTGTGCACACCTG
hCLCNKBex12	12	TGTCCACGCCTTGCCCAGCAG	CTTTCAGATACCTCCGGACCCCAG
hCLCNKBex13	13	CCTCAGGGATGGAGGGCTGTG	CACGACATTGCCCACGCAGCAG
hCLCNKBex14*	14	CTGCCTGACTCTGCCCTTGCAG	GACTCAGCCTGAGGTGGGCAC
hCLCNKBex15	15	GACTGTGGGGCCTGATGGGAG	CCTACCCCCGACTTCCTCCTC
hCLCNKBex16	16	GAACAGTTCTTGGCTAAGTAGGTGCCCATCCCCATGCCCCTCAG	
hCLCNKBex17	17	GGAGGCCAGCCCTGCACCTG	CCAGAGGCCTCATGTGTCACAC
hCLCNKBex18*	18	GGGCACCTTCTACCCTCCAGTG	GTCTTCTCAGGCATAGGTTCCCTG
hCLCNKBex19	19	TAAATTCCCCCCGCACCTCCAC	AGGGTCTCAGCCCAACCTC
hCLCNKBex1-2*	1-2	CGTGAAGGCTCCTCAGGGAAC	CTCGGACCACACTCTCAACAG
hCLCNKBex6-9*	6-9	GCGTGGCCACAGTCTTTGGCG	TGGAGCTGAACCTATTGTTCCTG
hCLCNKBex17-19*	17-19	TCCAGGACATCTTGGCTGCAG	AGGGTCTCAGCCCAACCTC

FIG. 10A

```
1     cggcagccac agcaggagga ctgacagggg cctgatggag gagtttgtgg ggctgcgtga
61    aggctcctca gggaaccctg tgactctgca ggagctgtgg ggcccctgtc ccctcatccg
121   ccgaggcatc cgaggtggcc tggagtggct gaagcagaag ctcttccgcc tgggcgagga
181   ctggtacttc ctgatgaccc tcggggtgct catggccctg gtcagctgtg ccatggactt
241   ggctgttgag agtgtggtcc gagcgcacca gtggctgtac agggagattg gggacagcca
301   cctgctccgg tatctctcct ggactgtgta ccctgtggcc ctcgtctctt tctcttcggg
361   cttctctcag agcatcacac cctcctctgg aggttctgga atcccggagg tgaagaccat
421   gttggcgggt gtggtcttgg aggactacct ggatatcaag aactttgggg ccaaagtggt
481   gggcctctcc tgcaccctgg cctgtggcag caccctcttc ctcggcaaag tgggcccttt
541   cgtgcacctg tctgtgatga tggctgccta cctgggccgt gtgcgcacca cgaccatcgg
601   ggagcctgag aacaagagca agcaaaacga aatgctggtg gcagcggcgg cagtgggcgt
661   ggccacagtc tttggcgctc ccttcagcgg cgtcctgttc agcatcgagg tcatgtcttc
721   ccacttctct gtctgggatt actggagggg cttctttgcg gccacctgcg gggccttcat
781   gttccggctc ctggcggtct tcaacagcga gcaggagacc atcacctccc tctacaagac
841   cagtttccgg gtggacgttc ccttcgacct gcctgagatc ttctttttg tggtgctggg
901   gggtctctgc ggcatcctgg gcagcgctta cctcttctgt cagcgaatct tctttggctt
961   catcaggaac aataggttca gctccaaact gctggccacc agcaagcctg tgtactccgc
1021  tctggccacc ttggttctcg cctccatcac ctacccaccc agcgccggcc gcttcctagc
1081  ttctcggctg tccatgaagc agcatctgga ctcgctgttc gacaaccact cctgggcgct
1141  gatgacccag aactccagcc caccctggcc cgaggagctc gacccccagc acctgtggtg
1201  ggaatggtac cacccgcggt tcaccatctt tgggaccctt gccttcttcc tggttatgaa
1261  gttctggatg ctgattctgg ccaccaccat ccccatgcct gccgggtact tcatgcccat
1321  ctttgtctat ggagctgcta tcgggcgcct ctttggggag actctctctt ttatcttccc
1381  tgagggcatc gtggctggag ggatcaccaa tcccatcatg ccaggggggt atgctctggc
1441  aggggctgca gccttctcag gggctgtgac ccacaccatc tccacggcgc tgctggcctt
1501  cgaggtgacc ggccagatag tgcatgcact gcccgtgctg atggcggtgc tggcagccaa
1561  cgccattgca cagagctgcc agcctcctt ctatgatggc accgtcattg tcaagaagct
1621  gccatacctg ccacggattc tgggccgcaa catcggttcc caccgcgtga gggtggagca
1681  cttcatgaac cacagcatca ccacactggc caaggacacg ccactggagg aggtggtcaa
1741  ggttgtgacc tccacagacg tggccgagta tcccttggtg gagagcacag agtcccagat
1801  cctggtgggc atagtgcgaa gggcccagct ggtgcaggcc ctgaaggctg agcctccttc
1861  ctgggctcct ggacaccagc agtgtctcca ggacatcttg gctgcaggct gccccacaga
1921  accagtgacc ctgaagctgt ccccagagac ttccctgcat gaggcacaca acctctttga
1981  gctgttgaac cttcattccc tctttgtgac gtcgcggggc agagctgtgg gctgcgtgtc
2041  ctgggtggag atgaagaaag caatttccaa cctgacaaat ccgccagccc caaagtgagc
2101  cggcccagca agatgaaaca gggcacccca gctgccctgg tactgaggtt gggctgagac
2161  cct
```

FIG. 10B

```
1     meefvglreg ssgnpvtlqe lwgpcplirr girgglewlk qklfrlgedw yflmtlgvlm
61    alvscamdla vesvvrahqw lyreigdshl lrylswtvyp valvsfssgf sqsitpssgg
121   sgipevktml agvvledyld iknfgakvvg lsctlacgst lflgkvgpfv hlsvmmaayl
181   grvrtttige penkskqnem lvaaaavgva tvfgapfsgv lfsievmssh fsvwdywrgf
241   faatcgafmf rllavfnseq etitslykts frvdvpfdlp eifffvvlgg lcgilgsayl
301   fcqriffgfi rnnrfsskll atskpvysal atlvlasity ppsagrflas rlsmkqhlds
361   lfdnhswalm tqnssppwpe eldpqhlwwe wyhprftifg tlafflvmkf wmlilattip
421   mpagyfmpif vygaaigrlf getlsfifpe givaggitnp impggyalag aaafsgavth
481   tistallafe vtgqivhalp vlmavlaana iaqscqpsfy dgtvivkklp ylprilgrni
541   gshrvrvehf mnhsittlak dtpleevvkv vtstdvaeyp lvestesqil vgivrraqlv
601   qalkaeppsw apghqqclqd ilaagcptep vtlklspets lheahnlfel lnlhslfvts
661   rgravgcvsw vemkkaisnl tnppapk
```

FIG. 10C

Mutated Residue and CLCNKB gene location	Affected Codon	Consequence in CLCNKB Protein	Patient
$^{405}C \rightarrow T$	C<u>C</u>G → C<u>T</u>G	$Pro^{124} \rightarrow Leu$	BAR274
$^{644}G \rightarrow A$	<u>G</u>CG → <u>A</u>CG	$Ala^{204} \rightarrow Thr$	BAR205
$^{1080}C \rightarrow A$	G<u>C</u>T → G<u>A</u>T	$Ala^{349} \rightarrow Asp$	BAR282
$^{1328}T \rightarrow C$	<u>T</u>AT → <u>C</u>AT	$Tyr^{432} \rightarrow His$	BAR283
$^{1346}C \rightarrow T$	<u>C</u>GC → <u>T</u>GC	$Arg^{438} \rightarrow Cys$	BAR218
$^{1571}C \rightarrow T$	<u>C</u>AG → <u>T</u>AG	$Gln^{513} \rightarrow$ Stop Codon	BAR283

INTERNATIONAL SEARCH REPORT

Inter ›nal Application No
PCT/US 98/20777

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C12Q1/68 C07K16/18 G01N33/68 C12N15/12 C07K14/47

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C12Q C07K C12N G01N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
P,X	SIMON D ET AL: "Mutations in the chloride channel gene, CLCNKB, cause Bartter's Syndrome Type III" NATURE GENETICS, vol. 17, no. 2, October 1997, pages 171-8, XP002095156 see the whole document ---	1-22
A	SAITO-OHARA F ET AL: "Assignment of the genes encoding the human chloride channels, CLCNKA and CLCNKB, to 1p36 and of CLN3 to 4q32-33 by insitu hybridization" GENOMICS, vol. 36, no. 2, September 1996, pages 372-4, XP002095374 see the whole document --- -/--	1

[X] Further documents are listed in the continuation of box C. [ ] Patent family members are listed in annex.

° Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
3 March 1999	16/03/1999

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Osborne, H

2

Form PCT/ISA/210 (second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

Inter· ›nal Application No
PCT/US 98/20777

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
A	TAKEUCHI Y ET AL: "Cloning, tissue distribution and intrarenal localization of CLC Chloride channels in human kidney" KIDNEY INTERNATIONAL, vol. 48, no. 5, November 1995, pages 1497-503, XP002095157 see the whole document	1
A	SIMON D ET AL: "Genetic heterogeneity of Bartter's Syndrome revealed by mutations in the K+ channel, ROMK" NATURE GENETICS, vol. 14, no. 2, October 1996, pages 152-6, XP002095158 see the whole document	1
A	SIMON D ET AL: "The molecular basis of inherited hypokalemic alkaldosis: Bartter's and Gitelman's Syndromes " AMERICAN JOURNAL OF PHYSIOLOGY, vol. 271, no. (5 Pt 2), November 1996, pages f961-6, XP002095159 see the whole document	1

2